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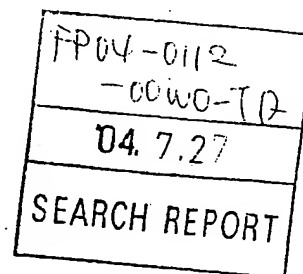
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(54) Title: LOW-LIPOXYGENASE 1 BARLEY

(57) Abstract: Barley plants having reduced lipoxygenase-1 enzyme activity are provided. For example, barley plants expressing mutant LOX-1 protein. The barley plants of the invention are useful in the production of plant products such as malt and brewed beverages, particularly beer, having increased stability and reduced T2N potential.



LOW-LIPOXYGENASE 1 BARLEY

This application is being filed as a PCT International patent application in the name of Carlsberg Research Laboratory, a Denmark corporation, on 29 December 2000, designating all countries except the
5 U.S.

FIELD OF THE INVENTION

This invention is in the field of plant biotechnology. More specifically, the invention relates to a mutant barley lipoxygenase 1 gene
10 (*lox-1*) that encodes an enzyme with severely reduced 9-hydroperoxy-octadecanoic acid forming activity. The invention also relates to the use of barley cultivars homozygous for *lox-1* in brewing processes to reduce the formation of off-flavors in brewed products, such as beer, during storage.

15

BACKGROUND OF THE INVENTION

Lipoxygenases are a family of enzymes (EC 1.13.11.12) that catalyze the dioxygenation of free and esterified poly-unsaturated fatty acids containing a 1(Z), 4(Z)-pentadiene configuration. The products of
20 lipoxygenase-catalyzed reactions have long been suspected as major culprits for the appearance of stale flavors in plant grain/seed and grain/seed derived food products (Robinson *et al.*, 1995, *Food Chem.*, 54: 33-43). Lipoxygenases have been implicated in the production of volatile hexanal aldehydes generated during soybean processing, which
25 have an undesirable aroma, limiting the use of soybean proteins in food products. Three lipoxygenase isozymes expressed in soybean seed are believed to contribute to lipid oxidation and hexanal formation. Soybean mutants lacking one or more of these isozymes have been generated with the aim of reducing hexanal formation and improving their flavor
30 stability. The success of this approach has been evaluated by Hildebrand *et al.*, 1990, *J. Agric. Food Chem.* 38: 1934-1936. Mutants lacking soybean lipoxygenase 3 produced higher hexanal levels, suggesting that

this isozyme diverts 13-hydroxyperoxyoctadecanoids, produced by lipid oxidation, towards non-volatile products. The field performance of triple-null soybean lines, lacking all three seed lipoxygenases, has shown that these enzymes are not essential for normal agronomic and seed characteristics (Narvel *et al.*, 1998, *Crop Sci.* 38: 926-928).

Lipoxygenases have also been implicated in the generation of off-flavors in rice, which can occur during grain storage. The release of free fatty acids can be detected in stored grain, which is indicative of the metabolism of the triglycerides reserves. The rice variety Daw Dam was found to accumulate lower levels of pentanals and hexanals giving a better flavor stability on storage (Susuki *et al.*, 1999, *J. Agric. Food Chem.*, 47: 1119-1124). This desirable phenotype was attributed to the absence of rice lipoxygenase-3, which oxidises unsaturated lipid acyl chains to form 9-hydroxyperoxyoctadecanoic positional isomers.

It is recognised that the lipoxygenase pathway is complex with many branches and its role in numerous aspects of plant growth and physiology are not fully understood. Modifications of the lipoxygenase pathway which alter 9-hydroperoxidation activity in seed crops are proposed to regulate their susceptibility to mycotoxin contamination by *Aspergillus* spp. (WO 9726364), which is consistent with the involvement of this pathway in plant pathogen resistance, but is not related to the aims of the invention herein described.

Among the many aroma volatiles which contribute to the flavor of beer, the higher unsaturated aldehydes with a 6-12 carbon chain have particularly low organoleptic flavor thresholds (Meilgaard 1975, *MBAA Tech. Quart.* 12: 151-168). *Trans*-2-nonenal, which is a member of this group, has both an extremely low flavor threshold of 0.11 ppb and contributes an unpleasant straw-like, "cardboard" flavor to the beer. The characteristic off-flavor caused by *trans*-2-nonenal is a common characteristic of beers stored for 1-3 months or more and is particularly detrimental to the flavor of lager beer, which is brewed with light malts and has a delicate flavor.

Sulfite has long been known to improve the flavor stability of beer, not only by binding oxygen and acting as an anti-oxidant, but also by forming volatile bisulfite addition compounds with aldehydes and ketones present in the beer. The two major sources of sulfite in beer are sulfite produced by yeast during fermentation via the sulfur assimilation pathway and sulfite added to the beer prior to packaging. Fermentation conditions that enhance yeast sulfite production and secretion will allow the formation of sulfite-carbonyl adducts from carbonyls present in the wort and prevent their further metabolism by the yeast (Dufour 1991, *Proc. Eur. Brew. Conv. Congr.*, Lisbon, pp. 209-216). In this manner carbonyls such as acetaldehyde and diacetyl may be transferred to the beer. The ability of sulfite to prevent the appearance of the carbonyl compound *trans*-2-nonenal during beer aging has been demonstrated by brewing beer with a yeast strain blocked in the sulfur assimilation pathway (Johannesen *et al.*, 1999, *Proc. Eur. Brew. Conv. Congr.*, Nice, pp. 655-662). Following bottling, the beer was subjected to forced aging by storing it at 37°C for 7 days, after which *trans*-2-nonenal levels were found to be well above the taste-threshold. If 10 ppm sulfite was added to the low-sulfite beer just prior to bottling, the appearance of *trans*-2-nonenal during forced aging was significantly reduced. The reaction between sulfite and carbonyl compounds is reversible and under thermodynamic and kinetic control. The apparent equilibrium constants for bisulfite compounds ranges from 10^{-6} M for carbonyl compounds such as acetaldehyde, hexanal, and decanal, to 10^{-3} for diacetyl and pyruvate (Dufour 1991, *supra*). During beer storage, gas exchange through the packaging will allow oxygen into the beer and sulfite will be lost, such that weaker bisulfite adducts will dissociate, allowing free carbonyls to appear in the beer. While sulfite unquestionably enhances the flavor-stability of beer, particularly in the short-term, its retention in packaged beer is strongly dependent on gas exchange through the packaging and temperature. In a finished beer the natural levels of sulfite produced during fermentation are variable and the addition of sulfite prior to

bottling is not a universally accepted practice. For these reasons sulfite alone does not provide a reliable method to enhance the long-term flavor-stability of beer under the different beer storage conditions used around the globe.

5 It is generally accepted that the *trans*-2-nonenal found in beer results from the oxidation of polyunsaturated fatty acids derived from barley grain lipids, where the 18-carbon chain fatty acid, linoleic acid [classified as an 18:2,n-6 polyunsaturated fatty acid (Broun, Gettner and Sommerville 1999, *Annu. Rev. Nutr.* 19: 197-216)] is the most abundant.
10 However, there is little agreement in the literature as to the mechanism whereby *trans*-2-nonenal is formed. The presence of enzymatic pathways leading to *trans*-2-nonenal formation from poly-unsaturated fatty acids has been proposed, but the individual enzymatic steps have never been demonstrated experimentally in barley grain or during the
15 malting process (Gardner 1988, *Adv. Cereal Sci. Technol.* 9: 161-215). The concept of using anti-sense or co-suppression gene technology to reduce lipoxygenase-1 levels in barley grain, and thereby control 9-hydroperoxidation and reduce aldehyde and alcohol levels in the finished barley grain, has been proposed as a means to control off-flavor
20 formation, but results of such an approach are not reported (McElroy and Jacobsen, 1995, *Bio/Technology* 13: 245-249).

 A forcing test has been developed as a method for assessing the *trans*-2-nonenal potential of a beer, where *trans*-2-nonenal formation in wort or beer is induced by subjecting samples to elevated temperatures at
25 reduced pH, (100°C, at pH 4.0 for 2 hours). Attempts to correlate the *trans*-2-nonenal potential in wort and finished beer with the total level of lipoxygenase activity in the kilned malt have indicated that lipoxygenase may contribute to the appearance of *trans*-2-nonenal in aged beer (Drost *et al.*, 1990, *J. Am. Soc. Brew. Chem.* 48: 124-131). The conclusions that
30 can be drawn from this study, however, are severely limited by the fact that the lipoxygenase activity in the barley malt was regulated at the end of the malting process by the degree of enzyme inactivation during kiln

drying. Thus, only the effect of the residual malt lipoxygenase activity on the *trans*-2-nonenal potential in the derived wort and finished beer was examined. The study failed to evaluate the lipoxygenases that catalyse the first step in the lipoxygenase enzymatic pathway in the barley grain during development and malting, and their role as determinants of *trans*-2-nonenal levels found in beer. Indeed, the absence of barley cultivars deficient in one or more lipoxygenase isoenzyme has made it impossible to provide convincing evidence for the role of the lipoxygenase pathway in barley malt in controlling the formation of *trans*-2-nonenal. Such experiments are needed to evaluate the contribution of enzymatic, as compared to auto-oxidative/chemical pathways, to the formation of *trans*-2-nonenal in beer. The brewing process involves a high temperature step of wort boiling where these non-enzymatic reactions are proposed to occur (Noël *et al.*, 1999, *J. Agric. Food Chem.* 47: 4323-4326).

SUMMARY OF THE INVENTION

This invention provides a barley cultivar having greatly reduced lipoxygenase-1 activity. In one embodiment, the barley plants of the invention contain a mutant *lox-1* gene expressing greatly reduced levels of the isoenzyme lipoxygenase-1. In an alternative embodiment, the barley plants contain a heterologous nucleic acid sequence expressing an antisense sequence to the wild-type *lox-1*, thereby reducing the enzyme's activity.

As shown herein, malt and wort produced from the reduced lipoxygenase barley of the invention, for example, from barley cultivars homozygous for a mutant *lox-1* gene, are useful to produce beer with significantly enhanced flavor stability and reduced *trans*-2-nonenal levels, particularly under conditions known to promote the appearance of T2N. The invention demonstrates a correlation between the activity of barley malt lipoxygenase-1 to produce 9-hydroxyperoxy-octadecadienoic acids (9-HPOD), and the presence of *trans*-2-nonenal in beer. The

invention further demonstrates that the use of barley homozygous for the mutant *lox-1* gene in the brewing process improves the flavor stability of the beer, both during storage and on exposure to elevated storage temperatures. These properties enhance the quality of the beer, and are
5 useful to extend its shelf-life and reduce the need to cool beer during transport and storage.

The invention provides barley plants and portions thereof having reduced lipoxygenase-1 activity, including barley plants expressing mutant LOX-1 protein as described herein, as well as methods for
10 producing such barley plants, plant portions, products of the plants, and particularly malt and beer products produced from the barley plants of the invention.

BRIEF DESCRIPTION OF THE DRAWINGS

15 Figure 1 is a graph showing the effect of the inhibitor nordihydroguaiaretic acid (NDGA) on immuno-affinity purified lipoxygenase 1 and 2 activity from embryos of 3 day germinated barley grain.

Figure 2 is a graph showing the fresh weight of developing grain
20 of Line G and cv Vintage from 5 days after flowering to full-maturity (FM). Each determination is the mean single grain weight from 6 spikes.

Figure 3 is a graph showing the dry weight of developing grain of Line G and cv Vintage from 5 days after flowering to full-maturity (FM). Each determination is the mean single grain weight from 3 samples of 5
25 grain.

Figure 4 is a graph showing total lipoxygenase activity in developing grain of Line G and cv Vintage from 5 days after flowering to full-maturity (FM).

Figure 5 is a graph showing 9- and 13-HPOD products of linoleic
30 acid oxidation by lipoxygenase activity in developing grain of Line G.

Figure 6 is a graph showing total lipoxygenase activity in embryos of germinating grain of Line G and cv Vintage expressed as μ mol/min/10 embryos (U/10 embryos).

Figure 7 is a graph showing 9- HPOD and 13-HPOD products of linoleic acid oxidation by lipoxygenase activity in embryos of germinating grain of Line G and cv Vintage, showing levels of 9- HPOD and 13-HPOD.

Figure 8 is a Western blot showing immunodetection of lipoxygenase 1 in embryos of developing grain of Line G and cv Vintage [wt] from 5 days after flowering to full-maturity (FM).

Figure 9 is a Western blot showing immunodetection of lipoxygenase 1 in embryos of grain of Line G and cv Vintage [wild-type] germinated for 0 - 6 days.

Figure 10 is a Northern blot probed with the 3' non-transcribed region of the *lox-1* cDNA and showing lipoxygenase 1 transcripts detected in developing grain of Line G and cv Vintage [wild-type] from 5 days after flowering to full-maturity (FM).

Figure 11 is a Northern blot probed with the 3' non-transcribed region of the *lox-1* cDNA and showing lipoxygenase 1 transcripts detected in embryos of grain of Line G and cv Vintage [wt] germinated for 0 - 6 days.

Figures 12A-12G are a nucleotide sequence alignment of the promoter and transcribed region of the *lox-1* wild-type cv Vintage allele (WT) and the Line G allele (LG). The transcription start site (+1), ATG start codon (+69) and translation stop codon (+4231) in the gene sequences are underlined. Nucleotide mutations identified in the Line G allele are shown in bold italics and indicated by an asterisk.

Figure 13 is a schematic presentation of the *lox-1* gene of cv Vintage (wild-type) and the mutant *lox-1* gene of Line G. The transcript from +1 to +4375 is composed of 7 exons (stippled boxes) and 6 introns (white boxes). Two mutations in the *lox-1* gene are indicated.

Figure 14 is a schematic drawing of gene cassettes for transient expression of the wild-type *lox-1* cDNA and *lox-1* gene and the mutant *lox-1* gene from Line G. The lipoxygenase coding sequences were cloned between the constitutive maize ubiquitin promoter with intron 1 (*Ubi-1*) and the *nos* terminator.

Figure 15 is a bar graph showing Lipoxygenase 1 activity in barley aleurone protoplasts transfected with gene cassettes containing the wild-type *lox-1* cDNA; the mutant *lox-1* gene from Line G; WT *lox-1* gene; and a control GUS reporter gene. Lipoxygenase activity in extracts of transfected protoplasts was assayed in microtiter plates by the oxidation of KI and quantitated spectrophotometrically. Lipoxygenase 1 activity was expressed as units per μg protein in the extract and is shown as the mean of 3 measurements from 2 replicate assays.

Figure 16 is a sequence alignment demonstrating that a RFLP between the wild-type and mutant *lox-1* gene is due to a point mutation at nucleotide 2347, creating an additional *Aat*II restriction site.

Figure 17 is a schematic presentation of the *lox-1* PCR fragments amplified and cleaved in the polymerase chain reaction – cleavage amplified polymorphic site (PCR-CAPS) assay. The positions of PCR primers are indicated by arrows and the *Aat*II sites are shown above the gene (sequence position). The exon and intron regions within the PCR product are distinguished by stippled and white boxes respectively, and the sizes of the *Aat*II digestion fragments are given.

Figure 18 is an electrophoretic agarose gel showing *lox-1* PCR fragments (652 bp) amplified in the first step of the PCR-CAPS assay from Line G and cv Vintage genomic DNA.

Figure 19 is an electrophoretic agarose gel showing RFLP detected by PCR-CAPS in the wild-type and mutant *lox-1* gene. The *Aat*II digestion fragments of the mutant gene include a unique 313 bp restriction fragment, indicated by an asterisk.

Figure 20 is a table showing a back-crossing program for the single recessive gene pair *ll* (low lipoxygenase trait) of Line G to cv

Alexis. The *LL* genotype are plants expressing wild-type lipoxygenase activity (dominant allele), the *ll* genotype are plants expressing the low-lipoxygenase (recessive allele). *Ll* are heterozygous plants containing both the wild-type and the low-lipoxygenase allele. Since the low-lipoxygenase trait is a recessive trait, *Ll* plants show wild-type lipoxygenase activity. After each round of back-crossing (including self-pollination), the *ll* progeny is expected to represent 25% of the progeny. The observed frequencies of low-lipoxygenase activity are indicated. The calculated percentage of the cv Alexis genetic background having the homozygous low-lipoxygenase allele is indicated as % Alexis.

Figure 21 is an electrophoretic agarose gel showing PCR-CAPS detection of the mutant *lox-1* gene in *ll* progeny of the Line G – Alexis back-cross program. PCR-CAPS assay on genomic DNA of Line G (Lane 2), cv Vintage (Lane 3), *ll* progeny of 3rd (Lane 4) and 4th back-cross (Lanes 5 – 9). DNA ladder (Lane 1). Control, backcrossed high *lox* line (lane 10).

Figures 22A-22B are a comparative alignment of amino acid sequences of soybean lipoxygenases LOX-1 (Gm1), LOX-2 (Gm2), LOX-3 (Gm3), and barley lipoxygenases LOX-1 (Hv1) and LOX-2 (Hv2).

DETAILED DESCRIPTION OF THE INVENTION

In accordance with the subject invention, plant materials, plant products, and methods are provided for producing a beverage, such as beer, the beverage having a reduced content of the off-flavor compound *trans*-2-nonenal, such that the flavor stability of the beverage, e.g., beer, during storage and on exposure to elevated temperatures is improved, relative to a control beverage. More particularly, the invention provides barley varieties whose developing and germinating grain produce greatly reduced activity levels of the enzyme lipoxygenase-1, denoted LOX-1, which, for example, when used in a beer brewing process, results in a

beer having reduced *trans*-2-nonenal levels, as compared with a control barley variety.

The methods used to generate, characterize, and validate a barley variety having greatly reduced LOX-1 activity, and use of this type of barley for the production of flavor-stable beer are described below.

1. Definitions

As used herein, the following terms have the indicated definitions:

10 "Plant portion" means a plant or specific part of a plant, such as the stem, leaves, roots, flowers, seeds, grains, fruits, or buds.

"LOX-1" means lipoxygenase-1 protein; "*lox-1*" means the gene encoding LOX-1.

15 "Mutant barley *lox-1*" means a mutagenized barley gene encoding a mutant lipoxygenase 1 polypeptide.

"Non-mutated control" means a plant, nucleic acid, gene, polypeptide, plant portion, or plant product containing wild type gene or protein.

20 "Heterologous" means a non-native sequence, e.g., a sequence derived from another species, or a recombinantly engineered or synthetic sequence that differs from the native sequence.

"Plant product" means a product resulting from the processing of a plant or plant portion, and includes, for example, malt and wort.

"Acidic amino acid" means aspartic or glutamic acid.

25 "Basic amino acid" means histidine, lysine, or arginine.

"Polar amino acid" means threonine, serine, tyrosine, tryptophan, asparagine, or glutamine.

30 "Organoleptic properties" means properties appealing to the olfactory and taste senses that are analysed, for example, by a trained taste panel.

"Brewed product" means a product prepared by mashing, boiling, and fermenting, e.g., beer.

"Reduced *trans*-2-nonenal" means less than about 50%, as compared with wild-type (control) conditions.

2. Lipoxygenase Activity

5 Lipoxygenase enzymes catalyze the oxidation of polyunsaturated fatty acids. In barley, the isoenzymes LOX-1 and LOX-2 are known. LOX-1 primarily catalyzes 9-hydroperoxidation, whereas LOX-2 primarily catalyzes 13-hydroperoxidation of polyunsaturated octadecanoic fatty acids. The data shown in the Examples below
10 demonstrates a correlation between barley LOX-1 9-hydroperoxidation activity and the presence of *trans*-2-nonenal in beer. Accordingly, barley having reduced LOX-1 activity is useful to produce beer having a reduced *trans*-2-nonenal level and/or potential as compared with a control.

15

3. Production of low lipoxygenase barley

A variety of known genetic approaches can be used to produce the plants of the invention, that is, to reduce the level of lipoxygenase 1 enzyme activity expressed in a barley plant in a stable, inheritable
20 manner. These approaches include, but are not restricted to antisense technology and mutagenesis, such as chemical and radiation induced mutagenesis, as well as site-directed mutagenesis.

Barley transformation. Barley can be transformed with various
25 nucleic acid molecules designed to manipulate *lox-1* gene expression or alter the architecture of the *lox-1* gene. Various methods, for example, *Agrobacterium tumefaciens*-mediated transfer (Tingay *et al*, 1997, *Plant J.*, 11: 1369-1376), particle bombardment (Wan and Lemaux, 1994, *Plant Physiol.*, 104: 37-48, or polyethylene glycol (PEG)-mediated DNA
30 uptake (Funatsuki and Kihara, 1995, *Theor. Appl. Genet.*, 91:707-712), can be used to successfully introduce nucleic acids into a barley cell, for example into a protoplast, callus, or an embryo.

Various promoters can be used to drive expression of the gene of interest. For expression of *lox-1*-containing vectors, including antisense sequences, the native *lox-1* promoter region can be used. The promoter sequence of *lox-1* is contained in nucleotides 2602 – 3511, which
5 includes the 5' UTR of EMBL accession no. U83904. Alternatively, promoters that drive expression of the gene of interest constitutively, for example the *Ubi.1* maize ubiquitin promoter, can be used (Wan and Lemaux, *Supra*; Kjærulff et al., in P. Mathis, Ed., 1995, *Photosynthesis: from Light to Biosphere*, Vol. II, 151-154). Expression vectors can also
10 contain a transcription termination region, for example, the 3' terminator of the nopaline synthase gene (3'-*nos*) (Bevan, *et al*, 1983, *Nucl. Acids Res.*, 11: 369-385) has been fused to genes expressed in transgenic barley (Wan and Lemaux, *Supra*; Funatsuki and Kihara, *Supra*).

Expression vectors can also contain a gene that allows for
15 selection of transformed cells when the vector has been successfully integrated in the cell. These genes can encode antibiotic or herbicide resistance genes, for example the neomycin phosphotransferase (*npt*) or the phosphinothricin acetyl transferase (*bar*) gene. When expressed, such resistance genes allow for growth of the transformed cell in neomycin -
20 or bialaphos-containing media, respectively (See, for example, Wan and Lemaux, *Supra*; Funatsuki and Kihara, *Supra*; Kjærulff et al., in P. Mathis, *Supra*).

Following transformation, cells can be grown in selective media for a period of time and then cultured to allow for the formation of
25 shoots, followed by root systems, and then plantlets. A successful barley transformation procedure was developed by Funatsuki and Kihara, (*Supra*), where transformation of barley protoplasts by PEG with neomycin phosphotransferase-containing expression vectors and subsequent selection in neomycin yielded fertile plants containing the
30 transgene. The transgene was shown to integrate into the genome and most of the transgenic plants expressed the protein encoded by the

transgene. These transgenic plants also were able to transmit and express the transgene following crosses.

It is understood that a variety of transformation methods, expression vectors, promoters, selectable markers, and the like are known and useful for transformation of barley.

Barley mutagenesis. The *lox-1* gene can be targeted for site-specific mutagenesis using chimeric RNA/DNA oligonucleotides. These chimeric RNA/DNA oligonucleotides have been shown to successfully introduce mutations in plant cells (Zhu et al., 1999, *Proc. Natl. Acad. Sci.* 96: 8768-8773; and Beetham et al., 1999, *Proc. Natl. Acad. Sci.* 96: 8774-8778) and mammalian cells (Yoon et al., 1999, *Proc. Natl. Acad. Sci.* 93:2071-2076) at desired locations. The chimeric RNA/DNA oligonucleotides can be transformed into the barley protoplasts or cells of interest in a variety of ways, for example using the PEG-mediated or particle bombardment-mediated transformation methods described above. The individual protoplasts or cells can then regenerated by tissue culture to whole fertile plants, and the mutational event can be confirmed and followed, for example using a PCR-based approach as detailed in the Examples below.

This site-directed mutagenesis method can be applied to mutate specific residues in the *lox-1* gene. The *lox-1* gene can be mutated at one or more nucleotide position in the promoter region to downregulate or abolish *lox-1* transcription. Specific mutagenesis can also be applied to introduce changes in the *lox-1* coding region that, for example, reduce the enzyme's activity. Such mutations include, but are not limited to, insertions, deletions, and substitutions resulting in a frameshift, truncation of the LOX-1 protein, and/or alteration of the neutral and hydrophobic nature of the enzyme's substrate cavity.

Antisense expression. Reduction in *lox-1* expression can also be accomplished by expression of a *lox-1* antisense construct in the barley cells. Methods for the expression of antisense constructs in barley to reduce the expression of a targeted protein have been reported, for

example, in Gilpin, M.J. et al., 1998, In: *Photosynthesis: Mechanisms and Effects*, G. Garab, ed., Vol. IV, 2983-2986; Kjærulff et al., 1995, In: *Photosynthesis: from Light to Biosphere*, P. Mathis, Ed., Vol. II, 151-154.

5 Barley cells can be transformed with an expression construct containing an antisense nucleic acid sequence. The expression construct produces an antisense RNA molecule capable of specifically binding to at least a portion of the mRNA produced from the wild type *lox-1* gene, through complimentary base pairing, and capable of disrupting the
10 splicing of the pre-mRNA or translation of this mRNA. A constitutive or tissue/temporal specific promoter, for example, the barley *lox-1* promoter described above, can drive expression of the antisense nucleic acid sequence.

Chemical mutagenesis. The chemical mutagen sodium azide.
15 (NaN_3) has commonly been used for barley mutagenesis and is known to induce stable mutations in the DNA (deoxyribonucleic acid) sequence of the barley genome (Olsen *et al.*, 1993, *Proc. Natl. Acad. Sci. USA*, 90: 8043-8047). Other chemical mutagens, for example, ethyl
 methanesulfonate (EMS), azidoglycerol (AG, 3-azido-1,2-propanediol),
20 methyl nitrosourea (MNU), and maleic hydrazide (MH) can also be used to induce DNA mutations (Rank, J. et al., 1997, *Mutat. Res.* 390:121-7), as can UV irradiation.

 As shown in the Examples below, the grain of the barley cultivars (cv) Vintage and Caruso were treated with sodium azide and propagated
25 by self-fertilization through to the 3rd generation (M3).

4. Identification and Selection of Low Lipxygenase Barley

Identification and selection of barley plants having reduced lipxygenase isoenzyme activity in the grain can be achieved, for example, by analysis of lipxygenase activity. Enzymatic assays can be used to determine the activity of the two major lipxygenases known to be present in either mature or germinating grain, LOX-1 and LOX-2. Such assays should distinguish LOX-1 activity from that of LOX-2.

One selective assay of LOX-1 and LOX-2 is based on the oxidation of a poly-unsaturated fatty acid by lipxygenase and the spectrophotometric detection of the hydroperoxide product of such oxidation. The specificity of this assay for LOX-1 takes advantage of the comparative insensitivity of LOX-1 to an inhibitor, for example, NDGA, relative to LOX-2.

Selective assay can also be achieved using immunoprecipitation to selectively remove LOX-1 or LOX-2 from the assay. Specific anti-LOX-1 and anti-LOX-2 antibodies, for example, monoclonal antibodies, can be prepared from purified LOX-1 or LOX-2 as described in Holtman et. al, 1996, *Supra*.

These assay methods can be adapted for microtiter plate assay procedures, or other known repetitive, high throughput assay formats, allowing the rapid screening of many samples. These assays can be validated for screening leaf tips of germinating grain in a non-destructive manner, such that seedlings selected in the screen can be further propagated.

The loss of LOX-1 activity in putative mutants can be confirmed by assay of enzymatic activity. For example, grain extracts can be incubated with linoleic acid and the oxidation products of linoleic acid analyzed, for example, by reverse phase HPLC. The relative amounts of 9-HPOD and 13-HPOD formed from linoleic acid provides a measure of LOX-1 activity, whose major product is 9-HPOD.

As shown in the Examples below, approximately 20,000 grain of the M3 generation of mutagenized cv Vintage and cv Caruso were screened for LOX-1 and LOX-2 activity by oxidation assay in the presence of inhibitor and also by immunoprecipitation assays. Using these screening methods, a mutant in cv Vintage was found having a major reduction in LOX-1 activity, and was denoted Line G. The mutant phenotype was inherited in the M4 and M5 generations.

Seed produced from the Line G barley was deposited on December 19, 2000 with the National Collections of Industrial, Food and Marine Bacteria (NCIMB), 23 St. Machar Drive, Aberdeen, AB243RY, Scotland, UK, under the terms of the Budapest Treaty, as Accession Number: _____.

5. Genetic Sequences

A precise description of the genotypic alteration that accounts for the low-lipoxygenase phenotype in barley plants of the invention is useful for identifying plants having this genetic alteration and for crossing this genetic character into other barley cultivars in a breeding program. A variety of known molecular and biochemical methods can be used to determine the genetic basis for the low lipoxygenase phenotype.

It is generally recognized that both cis-acting and trans-acting genetic sequences can determine the expression of a given gene in the genome and the activity of the gene product. Control points in gene expression include the regulation of the timing, tissue-specificity and rate of gene transcription, the stability of the transcript and the rate of transcript translation. Both the level of gene expression and the stability and specific activity of the encoded enzyme will determine the level of enzyme activity detected in a tissue.

Alterations in a plant gene sequence can be determined by DNA sequencing of known relevant parts of the genome, while Northern analysis provides a tool to monitor stable transcript levels in a given plant

tissue. Enzyme expressed in plant tissue can be evaluated by extracting the enzyme from the tissue and measuring the enzymatic activity.

As shown in the Examples below, the identity of the genetic changes that determine the low-lipoxygenase phenotype of the Line G mutant induced in cv Vintage were determined in the following manner. The structural gene encoding the LOX-1 protein, both in the parent cv Vintage and in the Line G, was amplified by the polymerase chain reaction (PCR), and the upstream promoter sequences, which regulate expression of the gene, as well as the entire coding sequence, comprising intron and exon sequences, were sequenced.

Comparison of the nucleotide sequences of the *lox-1* gene from Line G and from wild-type cv Vintage revealed 2 nucleotide substitutions in 2 exons, of which one (at position + 2347) led to a non-conservative amino acid substitution (Glycine³⁶⁸ → Aspartate) in the expressed protein.

Figure 22 shows an alignment of soybean (Gm: *Glycine max* L) lipoxygenases LOX1 (Acc. No. P08170), LOX2 (Acc. No. P08170), LOX3 (Acc. No. AAB41272) and barley (hv: *Hordeum vulgare*) lipoxygenases LOX1 (Acc.No. P29114) and LOX2 (Acc. No. AAB70865.1). Conserved amino acid residues and conservative substitutions of charged residues are shown in bold. Secondary structure assignments for LOX3 of soybean *Glycine max*, where H=alpha helices and E=Beta strands, are shown above the alignment, and residues relevant to enzyme function (identified by an asterisk or filled circle) are shown, as described in Skrzypczak-Jankun *et.al.*, 1997, *Proteins* 29:15-31.

Amino acid residues that participate in non-heme iron binding or essential for catalysis (*) in soybean LOX3 include: H₅₁₈, H₅₂₃, H₇₀₉ [3 N atoms]; N₇₁₃, I₈₅₇. The equivalent residues in barley LOX 1 are H₅₁₇, H₅₂₂, H₇₀₈, N₇₁₂, and I₈₆₂. Residues in soybean LOX3 with a predicted role in catalysis(•) are: H₂₆₆, H₅₁₃, H₇₇₆, F₂₆₄, F₂₇₂, F₇₁₄, W₅₁₉, R₅₅₂, R₇₂₆, D₇₆₆, D₇₇₉, K₂₇₈. The equivalent residues in barley LOX1 are: H₂₆₁, H₅₁₂, H₇₇₅, F₂₅₉, F₂₆₇, F₇₁₃, W₅₁₈, R₅₅₁, R₇₂₅, D₇₇₈, and K₂₇₃.

Proline (P_{86, 109, 167, 171, 223, 234, 291, 311, 324, 343, 345, 371, 381, 382, 486, 541, 548, 600, 616, 627, 685, 726, 734, 788, 829, 833, 839, 857}) and glycine (G_{49, 67, 68, 70, 91, 107, 137, 187, 192, 210, 217, 218, 260, 306, 307, 336, 392, 409, 458, 474, 490, 569, 607, 674, 676, 720, 736, 783, 828, 850, 855}) residues (+) located in loops and helix-capping positions in protein secondary structures, may facilitate sharp turns and folding of the peptide backbone.

Alignment of related plant lipoxygenases indicated that the Glycine-368 in barley LOX-1 is strongly conserved. Furthermore, this residue, which corresponds to Glycine-353 in soybean LOX-1, is one of 35 highly-conserved residues out a total of 58 residues that line the substrate cavity II of the enzyme, as seen from its crystal structure. These conserved residues are highlighted (boxes) in the alignment of plant lipoxygenase sequences shown in Figure 22 (Minor *et.al.*, 1996, *Biochemistry* 35:10687-10701), and include the following barley LOX-1 residues: Y₂₂₄, L₂₆₈, W₃₅₅, E₃₆₄, G₃₆₈, V₃₆₉, N₃₇₀, I₃₇₄, L₄₂₄, L₄₉₉, K₅₀₁, A₅₀₂, V₅₀₄, D₅₀₈, S₅₀₉, H₅₁₂, Q₅₁₃, L₅₁₄, H₅₁₇, W₅₁₈, H₅₂₂, I₅₅₆, L₅₅₉, A₅₆₀, L₅₆₄, I₅₆₅, I₅₇₀, T₅₇₄, S₅₈₅, Q₇₁₅, Y₇₁₈, N₇₂₄, R₇₂₅, P₇₂₆, T₇₂₇, L₇₇₂, and I₈₆₂. All but 7 of the 35 conserved residues are neutral or hydrophobic residues. The substitution of a charged residue at position Glycine – 368 in barley or at another conserved neutral or hydrophobic residue lining the substrate cavity II, is likely is likely to disturb the structural and functional properties of the enzyme. The G→D₃₆₈ mutation in barley Line G LOX1(♦) is located between alpha-helix H6 and beta-strand E12.

As shown in Figure 22, the lipoxygenase family of enzymes shares a high degree of sequence conservation, which is reflected in their conserved secondary structure, determined for several members of the plant lipoxygenase family including soybean LOX1 and LOX3 (Skrzypczak-Jankun *et al.*, 1997, *supra*). Barley LOX1 shares 56.9% sequence identity and 67.8% sequence similarity with soybean LOX3. Several amino acid residues in the soybean LOX3 isoenzyme have been identified as ligands for the non-heme iron, or are suggested to be essential for its activity (denoted by * •). In view of the high sequence

conservation between the barley LOX1 and the soybean LOX3, it is reasonable to predict that residues in the barley LOX1 sequence that are homologous to those identified as important for the function of LOX3 may also be essential for enzymatic activity. Thus, non-conservative amino acid substitutions at any of these positions, including substitutions of those residues in barley LOX1 corresponding to the 35 highly conserved residues of soybean LOX3 that line the substrate cavity and in other positions essential for enzyme activity, are likely to reduce lipxygenase activity.

The amino acid residues proline and glycine are known to facilitate turns in a peptide backbone when they are located between secondary structural elements, which allow a protein to assume a folded tertiary structure. Proline and glycine residues are also common in helix capping motifs (Parker and Hefford, 1997, *Protein Eng.*, 10: 487-496, <http://www.expasy.ch>). The single non-conservative substitution in Line G LOX1, where a glycine located between two predicted structural elements was replaced by aspartate, led to a significant loss of enzyme activity. It is thus predicted that mutation in the *LOX-1* gene causing a non-conservative amino acid substitutions at one or more of the proline or glycine residues in the barley LOX1, located in regions outside the structural elements, may similarly prevent folding of the native protein and consequently reduce the activity of the encoded enzyme.

Thus, in one embodiment, a useful mutant barley plant of the invention having reduced lipxygenase 1 activity contains a mutated nucleic acid sequence that alters the neutral or hydrophobic nature of the substrate cavity of the enzyme by insertion of one or more acidic, basic, or polar amino acids. For example, a useful nucleic acid sequence [SEQ ID NO: 11 encodes a barley LOX-1 protein [SEQ ID NO: 12] having a substitution at amino acid 368 from Glycine to Xaa, where Xaa is an acidic, basic, or polar amino acid. One specific amino acid sequence of the barley mutant LOX-1 of the invention is that where Xaa is aspartic acid, e.g., Line G.

As shown in the Examples below, the genotypic changes in Line G had no detectable influence on *lox-1* gene expression, but the LOX-1 activity detected in mature and germinating grain of Line G were approximately 9% of that detected in grain of the parent line, cv Vintage.

5 In order to provide direct evidence that the amino acid mutation in LOX-1 of Line G was responsible for the low-LOX-1 phenotype, the coding sequence of Line G *lox-1* and cv Vintage *lox-1* were expressed transiently in protoplasts from barley aleurone, and the activity of the mutant LOX-1 enzyme was shown to be strongly reduced in comparison to the wild-type

10 LOX-1 enzyme.

6. Transfer between breeding lines

The detection of alterations in genetic character of the barley plants of the invention genotype is useful to identify the presence of a

15 specific genetic character in a barley line, and to facilitate the transfer of this character between breeding lines in a breeding program. A variety of molecular tools are available for the detection of alterations in genomic sequence. Such methods include, but are not restricted to, detection of restriction fragment length polymorphisms (Gebhardt and

20 Salamini 1992, *Int. Rev. Cytology.*, 135: 201-237) and quantitative PCR based detection methods such as amplification using fluorescent primers, e.g. the TaqMan primer probe systems (Ibrahim *et al.*, 1998, *Anal. Chem* 70, 2013-2017). The choice of detection method will depend on the specific genetic character but should preferably be rapid and provide

25 clearly interpretable data.

As shown in the Examples below, a PCR-Cleavage Amplified Polymorphic Site assay (PCR-CAPS) was provided for the detection of the mutant lipoxygenase-1 gene of Line G. The nucleotide substitution in the *lox-1* gene in Line G at position + 2347 introduced an additional

30 site of recognition by the *AatII* restriction endonuclease that can be detected by the PCR-CAPS assay. Suitable detection methods for *lox-1*

are not restricted to this assay, but can equally well be based on TaqMan technology, and other known detection methods.

Also shown in the Examples below, the PCR-CAPS assay was applied to 4 generations of breeding material from a back-cross program, where the low-lipoxygenase phenotype in Line G was systematically back-crossed into cv Alexis. Inheritance of the low-lipoxygenase phenotype was shown to follow the inheritance of the *lox-1* gene, and the phenotype was identified as recessive and only seen in lines homozygous for the *lox-1* gene.

Accordingly, plant progeny of the invention includes breeding lines, for example, derived in a back-crossing program, that contain mutant *lox-1* and express a low lipoxygenase phenotype.

7. Brewing

The barley plants of the invention, including plant parts, plant progeny, grain, and plant products such as malt and wort, having low lipoxygenase 1 activity, are demonstrated herein to be useful for the manufacture of a beverage having reduced levels of free *trans*-2-nonenal over a measured period of time, or under conditions of elevated storage temperature, as compared to a beverage produced from a wild-type control barley variety. For the purpose of these comparisons the sulfite content of the beer is controlled to 5ppm or below, since it is recognised that higher sulfite levels at the time of bottling will temporarily delay the appearance of free *trans*-2-nonenal. For example, beer brewed from malt derived from the mutated barley Line G described herein, possessed stabilized organoleptic properties over a measured period of time as compared with beer brewed from malt derived from a control, non-mutated barley.

Brewing trials and evaluation of bottled beer provide the best method for evaluating the influence of different ingredients on the quality and stability of the finished beer. In order to test the influence of different barley malts, sufficient barley grain is needed to perform the

malting and brewing trials on a pilot scale and semi-industrial scale.

During the period of barley propagation, the field performance of the barley line can be evaluated. The malting properties of a barley line can be evaluated during pilot or industrial scale malting, and should

5 preferably lie within national malting quality recommendations eg. the European Brewing Convention recommendations for malting quality (Analytica-EBC/European Brewing Convention, 1998, Publ. Hans Carl Getränke-Fachverlag, Nürnberg, Germany). Following pilot or semi-industrial scale brewing, the beer is packaged in brown bottles and cooled
10 to 5°C for optimal storage. At this stage the fresh beer can be analysed by trained taste panels able to detect specific beer flavors, including the off-flavor compound *trans*-2-nonenal. Additionally, the beer is chemically analysed for major flavor components including *trans*-2-nonenal. These methods of beer quality analysis are then repeated on the
15 beer following various storage conditions known to reveal the long-term storage stability of the beer, for example, forced aging treatments.

As shown in the Examples below, Line G barley was propagated in the field over several seasons in order to malt 10 tons of this line in an industrial malthouse. The control barley varieties cv Vintage and cv
20 Nevada, both having the wild-type LOX-1 phenotype, were malted under similar conditions. The kilned malt from Line G and the control barley cultivars lay within the specifications required for the semi-industrial brewing trials.

Brewing trials were performed on a 30-hl scale and evaluation of
25 the freshly bottled beers revealed that beers brewed from malt of both Line G and the control cultivars had a *trans*-2-nonenal content below the taste-threshold and were deemed satisfactory by a taste-panel. Two forced-aging treatments, either storage at 37°C for 7 days or storage for 6 to 12 weeks at 30°C, were used to evaluate the flavor-stability of the beer.
30 The flavor-stability of beer brewed from Line G malt were found to be superior to that of control malt, both with respect to taste panel

evaluation as well as the level of free *trans*-2-nonenal, and the improvement was found to be statistically significant.

EXAMPLES

5 The present invention is further defined in the Examples below. It should be understood that the Examples, while indicating preferred embodiments, are given by way of illustration only.

Example 1

10 Screening and Selection of Lipoxygenase Isoenzyme Mutants from Mutagenised Barley

1. Barley mutagenesis

 Grains of barley, *Hordeum vulgare* cv Vintage and cv Caruso,
15 were mutagenised with sodium azide according to a published procedure (Kleinhofs *et al.*, 1978 *Mutation Research* 51: 29-35). The mutagenesis introduces point mutations in the genomic DNA that, for example, may result in amino acid changes in encoded proteins. The mutated M1 grains were propagated in the greenhouse through two generations, and
20 the M3 grain collected for screening. The observed frequency of single gene trait mutants in the M2 generation, according to Kleinhofs *et al.*, 1978, *supra*, are 1.0-2.7 mutants per 10,000 grain from the M2 generation. Since most gene mutations are recessive and only detectable in the homozygous state, the mutagenized population was screened at the
25 M3 generation where the expected proportion of homozygous mutant grain would be higher. A mutation frequency of 0.9 – 2.3 per 10,000 grain was expected in the mutagenized material at M3.

2. A non-destructive assay of lipoxygenase 1 (LOX-1) and 30 lipoxygenase-2 (LOX-2) activity in M3 mutagenized grain

 A rapid screening procedure for detection of mutant barley grain with reduced LOX-1 activity was developed with the following criteria:

The screening procedure should not prevent propagation of the grain/seedling; the selected grain/seedling tissue should express quantifiable levels of lipoxygenase activity; the assay should distinguish LOX-1 activity from that of LOX-2; and the assay procedure should encompass multiple samples.

The levels of total lipoxygenase activity in different tissues of the germinating grain, namely the shoot, root, and scutellum tissue of embryo and the endosperm were assayed as follows: Extracts of barley seedling tissue were prepared by homogenising the tissue in ice-cold 20 mM Tris-HCl, pH 7.5, containing 2 mM NaN₃ and 0.5 mM phenylmethylsulfonyl fluoride (PMSF), followed by removal of insoluble material by centrifugation at 1000 g for 10 minutes. Lipoxygenase activity in 100 µl extract was assayed at 25°C, by addition of 2.9 ml of 20 mM linoleic acid substrate, prepared by dispersing 35 µl linoleic acid (free acid, L-1376, Sigma, USA) in 5 ml H₂O containing 1% Tween 20. The reaction was followed spectrophotometrically, where the rate of increase in absorbance at 234 nm ($A_{234\text{nm}}$), due to the formation of conjugated diene in the hydroperoxide product, is proportional to the enzyme activity present. One unit of lipoxygenase activity is defined as $\Delta A_{234} = 0.001$ per minute in a 3-ml reaction, equivalent to the oxidation of 0.12 µmole linoleic acid.

The leaf tissue of grain germinated for 4 days in the dark had the highest detected levels of lipoxygenase activity (Holtman *et al.*, 1996, *Plant Physiology* 111: 569-576). Leaf tips from 4-day seedlings were thus selected for the non-destructive lipoxygenase screening assay. The pH optimum of total barley lipoxygenase activity was tested between pH 4.5 and pH 9.0 and found to be pH 6.5. Hence a 25 mM HEPES buffer (pH 6.5) containing 0.2 M boric acid was selected for the screening assay.

Since both LOX-1 and LOX-2 enzymes were immuno-detected in shoots of 4-day seedlings (Holtman *et al.*, 1996, *supra*), a LOX-1 and LOX-2 specific assay was used. The lipoxygenase inhibitor

nordihydroguaiaretic acid (NDGA), identified by Eskin *et al.*, 1977, *Crit. Rev. Food, Science and Nutrition* 9: 1-40, was found to be a selective inhibitor of barley lipoxygenases. NDGA at 1×10^{-5} M strongly inhibited purified barley LOX-2, while LOX-1 retained 47 % activity (Figure 1).

- 5 The selectivity of this inhibitor was tested in the leaf tip assay, by determining the ratio of 9-hydroperoxyoctadecanoid (9-HPOD) to 13-hydroperoxyoctadecanoid (13-HPOD), which result from linoleic acid oxidation by LOX-1 and LOX-2, respectively. In the lipoxygenase assay of cv Vintage leaf tips, the proportion of 13-HPOD formed fell from
- 10 24.5% to 9.5% on addition of $1 \cdot 10^{-5}$ M NDGA.

- A selective assay for LOX-2 activity in leaf tip extracts was based on the use of LOX-1-specific monoclonal antibody (5D2) (Holtman *et al.*, 1996, *supra*) to immunoprecipitate LOX-1 present in the extracts. The residual lipoxygenase activity detected in the extracts after LOX-1
- 15 precipitation provided a measure of LOX-2 activity. The efficiency of this immunoprecipitation (described below) was evaluated by quantifying the residual LOX-1 and LOX-2 in the extract supernatant by ELISA assay, using specific monoclonal antibodies against LOX-1 (denoted 5D2) and LOX-2 (denoted 5.8) (Holtman *et al.*, 1996, *supra*). LOX-1
- 20 immunoprecipitation from extracts of cv Vintage leaf tips removed 85% of (LOX-1) protein and 15% of LOX-2 protein.

- Immunoprecipitation was performed in a V-bottom 96-well plate by adding 5 μ l 5D2-coated Dynabeads (Dyna) and 75 μ l buffer [20 mM Tris-HCl pH 7.5, 1% v/v Bovine Calf Serum (HyClone)] to 20 μ l of each
- 25 leaf tip extract. The plate was incubated on a titerplate shaker (MTS4, IKA, Labor Technik) for 1 hour at 4°C. The immunoprecipitate was pelleted by centrifugation at 4°C in a Sigma 302-K centrifuge for 10 minutes at 2000 rpm. The supernatant (70 μ l) from each sample was assayed for lipoxygenase activity in a flat bottom 96 well plate, as
- 30 described below, but with addition of 100 μ l assay buffer (25 mM HEPES, 0.2 M boric acid, pH 6.5).

The LOX-1 and LOX-2 assays were adapted for a high-throughput screening method. Leaf tips (1 cm) from eight 4 day-germinated grains were individually homogenised in 150 µl ice-cold buffer (20 mM Tris-HCl, pH 7.5) for 2 x 30 seconds in a multi-well homogeniser (Berg *et al.*, 1992, *Electrophoresis* 13: 76-81). After centrifugation for 15 minutes at 3000 rpm, 40 µl of the supernatant of each extract was transferred to a flat bottom 96 well plate. To each well, 170 µl buffer (25 mM HEPES, 0.2 M boric acid pH 6.5, $1 \cdot 10^{-5}$ M NDGA) and 10 µl substrate (20 mM linoleic acid) were added and then incubated for 20 minutes at 25°C. The reaction was terminated by the addition of 20 µl saturated potassium iodide solution (KI) and incubated for a further 8 minutes at 25°C. The redox reaction between hydroperoxydienes and KI yields I_2 , which was monitored by its extinction maximum at 350 nm in a microplate reader (Multiskan MCC/340).

15

3. Identification of potential lipoxygenase 1 mutants in the M3 and M4 grain of mutagenised barley

Grain of the M3 generation of cv Vintage and cv Caruso was stored at 45°C for 6.5 days to break dormancy, ensuring a 95% germination frequency. M3 grain of cv Vintage (9318) and cv Caruso (9633) was germinated and screened for lines whose LOX-1 activity was 15% or less of wild-type grain. The putative mutant lines (50 cv Vintage and 42 cv Caruso lines) were propagated to the M4 generation, harvested, and the germinated grain re-screened. The mutant LOX-1 phenotype was confirmed in one cv Vintage line and six cv Caruso lines, after measuring the lipoxygenase activity in extracts of 5 leaf-tips from each line. When the LOX-1 and LOX-2 activities in germinating embryos of these 7 putative mutants were examined, only the cv Vintage mutant (denoted Line G) showed a major reduction in LOX-1. In mature quiescent grain, lipoxygenase activity present in the embryo is almost exclusively LOX-1 activity, due to the differential expression pattern of the two isoenzymes (Schmitt and van Mechelen, 1997, *Plant Sci.* 128: 141-150). The total

30

lipxygenase activity in extracts of embryos from Line G mature dry grain (M5 generation) was 0.06 ± 0.04 U/mg protein in comparison to 0.74 ± 0.44 U/mg protein in cv Vintage embryo extracts, as determined by the spectrophotometric lipxygenase assay described in section 2 of Example

5 1. The residual lipxygenase activity in mature embryos of Line G in both the M4 and M5 generations was found to be approximately 9 % of the parental line.

Example 2

10 **Line G is a cv Vintage Mutant with a**
Low-Lipxygenase Phenotype

The agronomic properties and mutant phenotype of Line G were analysed in material of the M5 generation. Initial analyses were

15 conducted to confirm that the analysed M5 material was homozygous for the mutant phenotype. The low LOX-1 phenotype in Line G, detected in the M3 generation, could result from a dominant or a recessive mutation. If the Line G selected at the M3 generation was heterozygous for a dominant mutation, then subsequent generations would show segregation

20 for the phenotype. The lipxygenase activity in 26 individual Line G embryos from quiescent grain of the M5 generation was measured and compared to cv Vintage wild type embryos. The lipxygenase activity in all Line G embryos was very low, with an average of 0.06 ± 0.04 U lipxygenase per mg protein, compared to 0.74 ± 0.44 U lipxygenase per

25 mg protein in wild type cv Vintage embryos. These data confirmed that Line G in the M5 generation was homozygous for the low lipxygenase trait.

1. Line G has a wild type plant growth physiology and grain development.

Line G and cv Vintage grain were germinated and grown in a climate chamber under 16 hours light at 15°C and 8 hours dark at 12°C at a relative humidity of 80%. The growth characteristics of Line G and cv Vintage plants were similar with regard to plant height, number of tillers per plant, the onset of flowering and number of grains per spike. The fresh weight (Figure 2) and dry weight (Figure 3) of grain of Line G and wild type cv Vintage during development from 5 days after flowering (DAF) until full maturity, approximately 90 DAF, were very similar.

2. Line G grain have a low-lipoxygenase 1 phenotype throughout development

Lipoxygenase activity was measured in extracts of developing barley grain of Line G (M5 generation) and wild type cv Vintage. Grain was homogenised in ice-cold 20 mM Tris-HCl buffer pH 7.5 containing 0.1% (v/v) Nonidet P-40, a non-ionic detergent that enhances lipoxygenase extraction, and centrifuged at 15,000 g for 20 minutes to remove insoluble material. Lipoxygenase activity in the extracts was measured polarographically in 200 µl oxygen-saturated buffer (0.2 M boric acid, 25 mM HEPES, pH 6.5) containing 1.2 mM linoleic acid at 25°C, using a Clark-type electrode to measure oxygen consumption. Lipoxygenase activity increased during the first 20 days of grain development in both Line G and wild-type grain, but only in Line G did the activity level fall during grain maturation (Figure 4).

The relative amounts of 9-HPOD and 13-HPOD formed during linoleic acid oxidation provides a measure of the levels of LOX-1 and LOX-2 activity in the grain extracts. In this case Nonidet P-40 was omitted from the grain extraction buffer to avoid the co-extraction of hydroperoxide-consuming enzymes. The extracts (100 µl), mixed with 10 ml 50 mM phosphate buffer pH 6.5 containing 200 µM linoleic acid, were incubated for 20 minutes. The reaction was terminated by adjusting

the pH to 3.5, and an internal standard was added. The hydroperoxides formed in the assay were bound on an octadecyl solid phase column (Bakerbond, Baker) and eluted with methanol. The 9-HPOD and 13-HPOD were then separated by reverse phase HPLC on a C-18 column with an isocratic elution solvent (tetrahydrofuran:Methanol:H₂O:acetic acid; 25:30:44.9:0.1 (v/v) adjusted to pH 5.5 with concentrated ammonia) at a flow rate of 0.5 ml/minute as described by Aarle *et al.*, 1991, *FEBS Letters* 280: 159-162. Hydroperoxides were detected at 234 nm and the HPOD peaks were corrected against the internal standard, prostaglandin B2.

Figure 5 shows that 13-HPOD was the major product of lipoxygenase activity present in grain during the first 20 DAF, while 9-HPOD was formed by lipoxygenases active during grain maturation. While both Line G and wild-type grain extracts shared a similar profile of 13-HPOD synthesising activity, Line G did not show the wild-type rise in 9-HPOD synthesising activity. These data are consistent with a loss of LOX-1 activity in maturing Line G barley grain.

3. Line G grain have a low-lipoxygenase 1 phenotype on germination

Total lipoxygenase activity in extracts of embryos of grain germinated at 15°C was assayed as described in Example 1. The lipoxygenase activity present in quiescent wild-type grain declined during the first 4 days of germination and then increased (Figure 6). In Line G, lipoxygenase activity in quiescent grain was very low but increased after 4 days.

Analysis of the HPODs formed by the lipoxygenase activity in germinating embryos showed that 9-HPOD was the major product of lipoxygenases present in quiescent wild-type grain (Figure 7). The level of 9-HPOD formation fell with the decline in lipoxygenase activity in the extracts. The rise in lipoxygenase activity after 4 days was accompanied by the formation of both 9-HPOD and 13-HPOD. The low lipoxygenase

activity in Line G quiescent grain was associated with an absence of HPOD formation, while the rise in activity after 4 days mainly produced 13-HPOD. These data provide evidence that LOX-1 activity leading to the formation of 9-HPOD is greatly reduced in the embryos of both
5 developing, quiescent and germinating barley grain of Line G, while LOX-2 activity leading to formation of 13-HPOD is unchanged in Line G.

Example 3

10 **Line G has a Mutant Lipxygenase 1 Gene (*lox-1*)**
Causing a Low Lipxygenase Phenotype

The molecular basis for the low-LOX-1 phenotype of Line G was investigated in order to provide a complete description of the mutant. The
15 following analyses were performed to provide a complete characterization of the phenotype:

1. Lipxygenase-1 is synthesised in the developing and germinating grain of Line G

20 Western blot analysis of extracts of embryos from developing and germinating barley grain were performed in parallel with the measurement of lipxygenase activity, as described in Example 2. The crude extracts were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) according to Laemmli,
25 1970, *Nature* 227: 680-685. The separated proteins were transferred to nitrocellulose by semi-dry blotting, according to Towbin *et al.*, (1979) *Proc. Natl. Acad. Sci. USA* 76: 4350-4354. The blot was probed with the LOX-1 specific monoclonal antibody, 5D2, as described Holtman *et al.*, 1996, *Plant Physiology* 111: 569-576, at 500x dilution, followed by
30 incubation with goat anti-mouse antibody coupled to alkaline phosphatase, and detected with the alkaline phosphatase substrates nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl-phosphate as described

by Holtman *et al.*, 1996, *Plant Physiol.* 111: 569-576. The Western analyses revealed that LOX-1 protein was detected in developing grain from 10 DAF in cv Vintage embryos and the level increased during grain maturation (Figure 8). The protein was also present in the embryo of cv Vintage quiescent grain but declined slowly during germination (Figure 9). Although LOX-1 is recognised in extracts of Line G embryos and migrates in SDS-PAGE as a protein of similar size to cv Vintage LOX-1, the immunodetectable levels of the protein in Line G were slightly lower than in cv Vintage.

2. The *lox-1* gene is expressed in the developing and germinating grain of Line G

Total RNA was isolated from embryos of developing and germinating barley grain, according to the procedure of Hensgens and van Os-Ruygrok, 1989, *Rice Genet. Newslett.* 6: 163-168, in parallel with the measurement of lipoxygenase activity, described in Example 2. The RNA samples (7.5 µg) were separated on denaturing agarose gels and Northern blotted as described by Sambrook *et al.*, 1989 in *Molecular Cloning, a Laboratory Manual*, Cold Spring Harbour Laboratory Press, NY. The blots were hybridised with a ³²P-labelled probe generated from the barley 3' untranslated region, nucleotides 2659-2801 [SEQ ID NO:1], of the *lox 1* cDNA (EMBL Accession no. L35931) as described by Holtman *et al.*, 1996 *Plant Physiol.* 111: 569-576, using the Amersham Random Prime Kit.

Lox-1 transcripts encoding LOX-1 were detected in embryos of developing and mature cv Vintage and Line G grain from 30 DAF (Figure 10). The level of *lox-1* transcripts increased during germination in both cv Vintage and Line G embryos, indicating *de novo* expression of the *lox-1* gene (Figure 11). Since the detectable levels of *lox-1* transcripts were similar in Line G and cv Vintage embryos, neither reduced *lox-1* transcription or transcript stability can account for the low-lipoxygenase phenotype of Line G.

3. The *lox-1* gene of Line G encodes a mutant form of lipoxygenase-1

The nucleotide sequence of the *lox-1* gene of Line G and cv Vintage were analysed and compared in order to determine the molecular basis for the low-LOX-1 phenotype of Line G, which is characterised by normal transcription of the *lox-1* gene, but reduced accumulation and activity in the expressed lipoxygenase enzyme in grain.

Genomic DNA from Line G and wild-type cv Vintage was isolated from seedling leaf tissue according to a method described by Pich and Schubert 1993, *Nucleic Acids Res.* 21: 3328. The *lox-1* gene in the genomic DNA preparations was amplified by polymerase chain reaction (PCR) using primers based on the sequence of the barley *lox-1* gene (van Mechelen *et al.* 1995, *BBA* 1254: 221-225; Rouster *et al.*, 1997, *Plant J.* 11: 513-523). The position and sequence of the oligonucleotide primers used to amplify the *lox-1* promoter and coding regions, indicated in Figure 12 were as follows:

Forward primer 5'-GAA AAG CTT GGA GGT AGA CGC TGC-3' [SEQ ID NO:2] and reverse primer 5'-TAT AGG ATC CTT GTT CTT GGC CTC CTC TCC TCG-3' [SEQ ID NO:3] were used to PCR amplify the *lox-1* promoter domain (-361 to +68) of Line G and cv Vintage *lox-1*.

Forward primer 5'-AGT GAA AAA CAG TGT GCT GGT G-3' [SEQ ID NO:4] and reverse primer 5'-GGC TTA AAG AGC AAC TGC TGA-3' [SEQ ID NO:5] were used to PCR amplify the Line G *lox-1* coding region.

Forward primer 5'-CAA GAT GCA TAT GCT GCT GGG AG-3' [SEQ ID NO:6] and reverse primer 5'-CGA TGG TTT AAA TTA GAT GGA GAT GCT GT-3' [SEQ ID NO:7] PCR amplified the cv Vintage *lox-1* coding region.

The PCR reactions consisted of 250 ng genomic DNA in a 50 µl volume containing 50 pmol primer and 2 U *Pfu* DNA polymerase (Promega) according to the enzyme suppliers instructions. The PCR

amplifications were carried out in a Stratagene Robocycler: 1 minute at 94°C, 1 cycle; 1 minute at 94°C, 2 minutes at 62°C, and 5 minutes at 72°C, 30 cycles; 10 minutes at 72°C, 1 cycle. The PCR products were separated on 1.2 % agarose gels. DNA fragments, corresponding in
5 length to the amplified region, were purified using Qiax II Gel extraction kit (Qiagen) and cloned into the plasmid pcDNA2.1 (Invitrogen). The nucleotide sequence of both strands of the cloned *lox-1* promoter and coding regions was determined using the dideoxynucleotide chain termination reaction with specific oligonucleotide primers and analysed
10 on an ABI PRISM® 310 Genetic Analyzer (PE Biosystems). Sequence comparisons were performed using the DNA STAR sequence analysis software package (DNA STAR Inc., USA).

The promoter region and intron-exon structure of the barley *lox-1* coding region are shown in **Figure 13**, and were deduced from a
15 comparison of the nucleotide sequence of the wild-type *lox-1* genomic and cDNA sequences (**Figure 12**). The sequenced region of the *lox-1* promoter region from -363 to +68, (numbered relative to the determined transcription start site; van Mechelen *et al.*, 1995, *BBA* 1254: 221-225), is sufficient to direct embryo-specific and temporally-regulated gene
20 expression characteristic of the native gene (Rouster *et al.*, 1998, *Plant J* 15: 435-440). The promoter and transcribed region of the wild-type *lox-1* gene [SEQ ID NO:8] is 4663 nt in length and contains 6 introns of between 82 nt and 634 nt in length, which are absent from the respective cDNA [SEQ ID NO:10] and must therefore be removed during RNA
25 transcript splicing.

Comparison of the nucleotide sequence of *lox-1* of Line G with that of wild-type (**Figure 12**) showed that the Line G *lox-1* allele has two point mutations. One is a silent C→T substitution at position 221 in exon 1, and the second is a G→A substitution at position 2347 in exon 3
30 (**Figure 13**). The wild-type barley *lox-1* gene encodes a protein of 862 amino acid residues [SEQ ID NO:9], while the mutation at position 2347

in Line G *lox-1* allele causes an amino acid substitution of glycine to aspartic acid at residue 368 in the encoded protein.

Alignment of related plant lipoxygenases indicated that the glycine-368 in barley LOX-1, is strongly conserved. Furthermore this residue, which corresponds to glycine-353 in soybean LOX-1, is one of 51 neutral or hydrophobic residues which line the substrate cavity of the enzyme, as seen from its crystal structure (Minor *et al.*, 1996, *Biochemistry* 35: 10687-10701) and is shown in (Figure 22). The insertion of a charged amino acid residue at this position is thus likely to disturb the structural and functional properties of the enzyme.

4. The mutated LOX-1 protein encoded by the Line G *lox-1* allele has low enzymic activity and is responsible for the low lipoxygenase phenotype of Line G.

The sodium azide mutagenesis of cv Vintage grain, which induced the mutated *lox-1* allele in Line G, may have induced additional mutations in the Line G genome. Two experimental approaches have been taken to demonstrate that the mutant *lox-1* allele in Line G is responsible for its low lipoxygenase phenotype, rather than other mutations in the genome. The enzymic activity of the LOX-1 encoded by the mutant and wild-type *lox-1* allele have been determined in order to prove that the glycine→aspartic acid substitution in the mutant enzyme causes reduced stability and activity. The two *lox-1* genes were transiently expressed in aleurone protoplasts isolated from imbibed mature grain, since the level of endogenous lipoxygenase expression in these cells was expected to be below detection limits. None of the identified barley lipoxygenase genes, which are expressed in germinating barley, are detected in the aleurone tissue (van Mechelen *et al.*, 1999 *supra*). In order to direct transient expression of the *lox-1* gene in aleurone protoplasts, their coding regions were translationally fused to a constitutive promoter known to be active in these protoplasts.

The coding regions of the mutant (sequence positions +1 to +4350) and the wild-type *lox-1* gene (sequence positions +69 to +4230) and the wild-type *lox-1* cDNA (sequence positions +69 to +2654) each cloned in plasmid pcDNA2.1 (see section 3), were excised by digestion of the *KpnI* and *EcoRV* sites in vector polylinker. The coding regions were cloned in the pUBARN plasmid (Jensen *et al.*, 1998, *Hereditas* 129: 215-225) between the constitutively active maize ubiquitin *Ubi* promoter (as described in U.S. patent No. 005510474A) and the *Nos* terminator, in place of the *bar* gene which encodes phosphinotricin acetyl transferase (Figure 14).

Protoplasts were isolated from aleurone tissue of imbibed *Hordeum vulgare* cv Himalaya according to the protocol of Skriver *et al.* 1991, *Proc. Natl. Acad. Sci. USA* 88: 7266-7270. Aliquots of $2 \cdot 10^5$ protoplasts were transfected at 0°C with ~100 µg plasmid DNA (equimolar amounts of each plasmid) by polyethylene glycol (PEG) mediated DNA uptake (Lee *et al.*, 1997, *Plant Mol. Biol.* 13: 21-29), and then incubated in aleurone protoplast culture media at 25°C as described previously (Skriver *et al.*, 1991 *supra*). After 48 hours incubation, the culture medium was carefully removed and the protoplasts were re-suspended and homogenised in 300 µl lipxygenase assay buffer (0.2 mM boric acid, 25 mM HEPES, pH 6.5). The homogenates were centrifuged at 15,000 *g* for 5 minutes to pellet insoluble material, and the supernatants (10 µl) were subsequently assayed for total lipxygenase activity using the rapid screening assay described in Example 1, section 1, but with omission of the NDGA inhibitor. The protein content of the protoplast extracts was measured by a Bradford dye-binding assay (Bradford 1976, *Anal. Biochem.*, 72: 248) supplied by Bio-Rad Laboratories, Hercules, California, USA, and lipxygenase activity was expressed per mg protein in the extract.

Protoplasts transfected with the control plasmid, pUBI-GUS, where the maize ubiquitin-1 promoter directs expression of the β -glucuronidase reporter gene, gave no detectable lipxygenase activity.

Transient expression of the wildtype *lox-1* gene and cDNA in protoplasts both gave high levels of lipoxygenase activity in the protoplast extracts (Figure 15). The higher expression of the wild-type *lox-1* cDNA in comparison to the genomic sequence may be due to a higher transfection frequency for the smaller *lox-1* cDNA expression plasmid (4929 bp versus the 6505 bp *lox-1* gene construct). Transient expression of the mutant *lox-1* gene gave low levels of lipoxygenase activity, ~10 % of wild-type lipoxygenase activity. These data clearly demonstrate that the mutant *lox-1* gene in Line G encodes a lipoxygenase with greatly reduced activity, which accounts for the low lipoxygenase phenotype.

Example 4

PCR-Cleavage Amplified Polymorphic Site (PCR-CAPS) Assay:

A Method Used for Identification of the Mutant *lox-1* Gene

15

An analytical method allowing the identification of the Line G mutant *lox-1* gene in any genetic background was developed based on the PCR-CAPS assay. The assay involves PCR amplification of genomic DNA fragments, followed by digestion of the amplified sequences with a specific restriction endonuclease to display a restriction fragment length polymorphism (RFLP).

The coding sequence of the mutant *lox-1* gene harbours two point mutations (see Example 3), where the mutation at position 2347 (Figure 12) introduces an additional *Aat* II restriction endonuclease cleavage site, not found in the wild-type *lox-1* gene (Figure 16). The following PCR-CAPS assay, based on the polymorphism created by the presence of this restriction site in the *lox-1* gene, is shown to discriminate between a wild-type *lox-1* gene and a mutated *lox-1* gene.

Genomic DNA was isolated from young leaves of M6 seedlings of *Hordeum vulgare*, L. cv Vintage and Line G according to the procedure of Pich and Schubert (1993, *supra*). The DNA sequence encompassing position 2347 (Line G *lox-1* gene mutation site) was

amplified by PCR, using primers specific for the *lox-1* gene [SEQ ID NO:8]. The DNA fragments amplified by the selected forward primer 5'-CGCTACGACGTCTACAACGA-3' [SEQ ID NO:13] and reverse primer 5'-CAGACTACTTTTTGGCGGGA-3' [SEQ ID NO:14] are shown in Figure 17. PCR reactions were carried out with 250 ng genomic DNA in a 50- μ l volume containing 50 pmol of each primer and 1 unit Taq DNA polymerase (Promega) according to the suppliers instructions. PCR amplifications were carried out on a Stratagene Robocycler as follows: 1 minute at 94°C, 1 cycle; 1 minute at 94°C, 1.5 minutes at 60°C, and 2 minutes at 72°C, 30 cycles; 10 minutes at 72°C, 1 cycle. The amplified fragments of the mutant and wild-type *lox-1* gene were ~650 bp (Figure 18), corresponding to the expected size (Figure 17). The PCR products, purified on a spin column (Qiagen), were digested with 25 unit *Aat* II restriction endonuclease for 24 hours at 37°C and analyzed on a 1.2% agarose gel.

Digestion of the wild-type *lox-1* PCR product yielded DNA fragments of 10, 179, and 462 bp, and the fragments from the mutant *lox-1* PCR product were 10, 149, 179, and 313 bp, where additional DNA fragments were due to partial digestion of the *lox-1* PCR product (Figure 19). The fragment pattern corresponds to the expected RFLP resulting from this mutation, where the 313 bp fragment is unique to the mutant *lox-1*. This PCR-CAPS assay provides a reproducible and specific tool for identification of the *lox-1* allele in barley and can thus be exploited in barley breeding programs aimed at introducing this gene in new barley varieties.

Example 5

Back-Crossing the Low Lipxygenase Phenotype of Line G to cv Alexis Demonstrates a Genetic Linkage to the Mutant *lox-1* Gene

Repeated back-crossing was used to transfer the low-lipxygenase phenotype from line G into a recurrent parent (in this case the cv. Alexis). The back-crossing program shown in Figure 20, combined with selection

for the low-lipoxygenase phenotype, progressively substitutes the Line G genome by the recurrent parent genome. Furthermore, other mutations introduced into the Line G genome during the sodium azide mutagenesis treatment will be eliminated. In the first back-cross of the homozygous low-lipoxygenase Line G (denoted genotype *ll*) to cv Alexis (denoted genotype *LL*) the progeny lines will be heterozygous (denoted genotype *Ll*). A low-lipoxygenase phenotype due to a recessive mutation will not be detectable in lines heterozygous for the mutation. The progeny are self-pollinated and will give a normal Mendelian segregating population, namely $1LL: 2Ll: 1ll$. The low lox homozygous *ll* genotype resulting from the first back-cross will have 50 % cv Alexis genetic background. After ten rounds of back-crossing, the recurrent parent background will be approximately 99.9 %.

Hordeum vulgare, L. cv Alexis and Line G were propagated in a greenhouse throughout the back-crossing program. Back-crossed progeny grains were germinated in petri dishes on filterpaper, soaked with 4 ml H₂O, for 3 days at 22°C in the dark. The low-lipoxygenase lines were screened by measuring total lipoxygenase activity in extracts of the coleoptile (top 7 mm) from the germinating seedlings, as described in Example 1. Progeny of the 3rd and 4th back-cross were also analysed for inheritance of the mutant *lox-1* gene using the PCR-CAPS assay described in Example 4.

The expected frequency of the low-lipoxygenase phenotype in the segregating progeny of the four back-cross generations was 25% for a recessive mutation. The observed frequency of low-lipoxygenase activity in the progeny (24 grains) of the four back-cross generations is in agreement with the expected frequency (Figure 20). When the 3rd and 4th back-cross progeny having the low lox homozygous *ll* genotype were analysed with the PCR-CAPS assay, they were all found to have the diagnostic 313 bp fragment, while progeny having wild-type lipoxygenase activity lacked this fragment (Figure 21).

The back-crossing program demonstrates that the mutant *lox-1* allele can be transferred to a new genetic background and is inherited in a recessive monofactorial manner following Mendelian segregation. Since the recurrent parent background is 93.8% in the 4th back-cross progeny, the co-inheritance of the mutant *lox-1* gene and the low-lipoxygenase phenotype provides confirmation of their genetic linkage.

Example 6

Beer Brewed From Line G Barley Malt Accumulates Less *trans*-2-nonenal During Storage, Giving an Improved Flavour Stability

10

Hordeum vulgare L cv Vintage and Line G were propagated in the field over several seasons in order to provide sufficient grain for industrial malting. The following industrial scale malting and brewing trials as well as analyses of the finished beer were performed to demonstrate the value of the Line G low-lipoxygenase barley for improved flavour stability.

15

1. Industrial malting and kilning of Line G and cv Vintage

Malting was performed on a 10-ton scale in an industrial malthouse in two trials as follows:

20

Trial 1: Line G barley grain (1996 harvest)

Steeping conditions: 8 hours wet; 14 hours dry; 8 hours wet; 10 hours dry; 4 hours wet in 16°C steeping water. Malting conditions: 12 hours at 18°C; 24 hours at 16°C; 24 hours at 14°C; 60 hours at 12°C. Kilning conditions: 12 hours at 60°C; 3 hours at 68°C; 4 hours at 74°C; 3 hours at 80°C.

25

Trial 2: cv Vintage and Line G (1996/1997 harvest)

Steeping conditions: 8 hours wet; 10 hours dry; 6 hours wet; 15 hours dry; 4 hours wet in 15°C steeping water. Malting conditions: 5 days with inlet air at 15°C and spraying to maintain moisture level. Kilning conditions: 10 hours at 50°C; 2 hours at 60°C; 2.5 hours at 80°C.

30

Malting analyses of 2 samples of the Line G malt from Trial 1 compared to the control malt, cv Nevada (Table 1) and from Trial 2 compared to cv Vintage (Table 2) confirmed that Line G malt was suitable for brewing trials.

TABLE 1

MALTING TRIAL 1				
Barley variety		cv Nevada	LineG	LineG
Crop year		1996	1996	1996
Malt analyses				
Moisture content	%	4.7	4.3	4.4
Extract fine as is.	%ai.	76.9	76.1	75.3
Extract fine d.m.	%dm.	81.4	79.5	78.7
Saccharification time	Min	< 10	< 10	< 10
Diastatic power	%WK	252	373	365
Color	EBC	2.8	4.4	3.8
pH		6.16	5.97	5.99
Turbidity	EBC	9.0	2.5	2.4
Total protein d.m.	%	10.35	10.74	12.03
Soluble nitrogen	mg/l	647	787	765
Sol. Protein % malt d.m.	%dm.	3.7	4.4	4.3
Kolbach		35.3	40.8	35.4
Free Amino Nitrogen	mg/l	97	125	118
Friability	%	89.5	85.6	89.5
Whole unmodified grains	%	1.1	0.6	0.5
Partly unmodified grains	%	2.3	1.0	0.6
β -glucan in wort	mg/l	114	66	36
β -glucan in malt	% w/w	0.24	0.11	0.05
S-methylmethionine/DMS eq.	μ g/g	2.4	6.4	8.4
Free DMS	μ g/g	1.0	6.6	4.7
NDMA	μ g/kg	n.d.	0.3/0.6	0.3/0.3

TABLE 2

MALTING TRIAL 2				
Barley variety		Vintage	Line G	Line G
Crop year		1996	1996	1997
Moisture content	%	4.1	4.1	4.3
Extract fine as is.	%ai.	77.0	75.6	77.5
Extract fine d.m.	%dm.	80.3	78.8	80.9
Fine/coarse difference	% dm.	0.7	1.6	1.7
Saccharification time	min	-	-	< 10
Diastatic power	%WK	343	342	268
Color	EBC	2.5	2.8	3.4
PH		6.05	6.01	6.12
Turbidity	EBC	1.5	1.3	2.5
Total protein d.m.	%	10.98	12.22	9.82
Soluble nitrogen	mg/l	696	741	610
Sol. Protein % malt d.m.	%dm.	3.9	4.1	3.4
Kolbach		35.2	33.7	34.6
Free Amino Nitrogen	mg/l	110	117	100
Friability	%	91.3	81.8	89.5
Whole unmodified grains	%	0.7	1.1	1.3
Partly unmodified grains	%	1.0	2.7	2.7
β -glucan in wort	mg/l	97	172	117
Beta-glucan in malt	% w/w	-	-	0.3
S-methylmethionine/DMS eq.	μ g/g	-	-	-
Free DMS	μ g/g	-	-	-

2. Industrial brewing with Line G, cv Vintage malt, and control malt cv Nevada

Two brewing trials were performed, using wort prepared from Line G and control malt cv Nevada malt in Trial 1 and from Line G and control malt cv Vintage in Trial 2.

Beer was brewed on a 30-hl industrial scale with 475 kg malt
5 according to the following scheme: Mashing in at 50°C; 30 minutes at 50°C; 30 minutes heating from 50 - 70°C; 15 minutes at 70°C. A portion of the wort was heated for 20 minutes from 70 - 100°C and 5 minutes at 100°C, while the main mash was kept at 70°C for another 25 minutes and then the two mashes was combined and kept for 10 minutes at 76°C. The
10 brewing steps of wort boiling, whirlpool separation of spent grain, cooling, fermentation, lagering and packaging in brown glass bottles were according to standard brewing practise.

3. Flavor stability and T2N content of beer brewed from Line G, cv 15 Vintage malt and control malt cv Nevada

The freshly bottled beer was stored at 5°C and analysed within 2 months of production. The flavor-stability of the fresh and stored beer was evaluated in two independent laboratories following two different types of beer storage conditions. In laboratory A the beer was subjected
20 to a forced aging process, where the beer was stored at 37°C for a period of 7 days, while in laboratory B the beer was stored at 30°C for 6 and 12 weeks. *Trans*-2-nonenal levels in beer were determined by gas chromatography and mass spectrometric detection following derivatisation of carbonyls with O-(2,3,4,5,6-pentafluorobenzyl)-
25 hydroxylamine, essentially as described by Grönqvist et al. 1993 *Proceedings of the 24th EBC Congress*, Oslo, 421-428. A trained beer taste panel evaluated the overall flavor score of the beer, which includes detection of a cardboard flavor, indicative of free *trans*-2-nonenal in the beer.

30 Laboratory A: Forced-Aging Tolerance

Comparison of beer, brewed from Line G and the control malt, cv Nevada, in the first brewing trial (Table 3) demonstrated that beer from

Line G had a greater flavor stability and a lower *trans*-2-nonenal content following forced-aging as compared to the controls. The second trial, comparing beer brewed from Line G malt with beer brewed from cv Vintage malt, the parental cultivar, confirmed the initial data (Table 4).

5

TABLE 3

BREWING TRIAL 1		
Barley Malt	cv Nevada	Line G
SO ₂ Content (mg/ml)	1	1
T2N** (ppb) – Fresh Beer	0.009	0.005
T2N** (ppb) – Aged Beer (37°C / 7 DAY)	0.117	0.025
Flavor* - Fresh Beer	5.9	5.3
Flavor* – Aged Beer (37°C / 7 DAY)	1.3	5.1

* Flavor evaluation scale 1-10 of increasing quality; ***trans*-2-nonenal.

TABLE 4

BREWING TRIAL 2		
Barley Malt	cv Vintage	Line G
SO ₂ Content (mg/ml)	2	2.5
T2N** (ppb) – Fresh Beer	0.023	0.019
T2N** (ppb) – Aged Beer (37°C / 7 Day)	0.078	0.035
FLAVOR* - Fresh Beer	5.5	6.1
FLAVOR* - Aged Beer (37°C / 7 Day)	2.9	5.9

* Flavor evaluation scale 1-10 of increasing quality; ***trans*-2-nonenal.

10

Laboratory B: 30°C Storage Tolerance

Beer brewed from Line G malt had lower *trans*-2-nonenal levels following 6 and 12 weeks at the elevated storage temperature of 30°C, when compared to beer brewed from either of the reference malts (Table 5 and 6) and had a better flavor-stability as judged by a taste panel. The taste-threshold for *trans*-2-nonenal in these analysed beers lies close to 0.08 ppb.

15

TABLE 5

BREWING TRIAL 1		
Barley Malt	cv Nevada	Line G
<i>trans</i> -2-nonenal (ppb) – Fresh Beer	0.050	0.044
<i>trans</i> -2-nonenal (ppb) – 30°C / 6 weeks	0.072	0.037
<i>trans</i> -2-nonenal (ppb) – 30°C / 12 weeks	0.095	0.046
<i>trans</i> -2-nonenal flavor* – Fresh Beer	0.6	0.3
<i>trans</i> -2-nonenal flavor* – 30°C / 6 weeks	3.7	1.4
<i>trans</i> -2-nonenal flavor* – 30°C / 12 weeks	2.5	0.6

* *trans*-2-nonenal flavor detection score on a scale of 1 -10

5

TABLE 6

BREWING TRIAL 2		
Barley Malt	cv Vintage	Line G
<i>trans</i> -2-nonenal (ppb) – Fresh Beer	0.070	0.062
<i>trans</i> -2-nonenal (ppb) – 30°C / 6 weeks	0.093	0.070
<i>trans</i> -2-nonenal (ppb) – 30°C / 12 weeks	0.133	0.080
<i>trans</i> -2-nonenal flavor* – Fresh Beer	0.3	0.9
<i>trans</i> -2-nonenal flavor* – 30°C / 6 weeks	2.5	1.7
<i>trans</i> -2-nonenal flavor* – 30°C / 12 weeks	2.2	1.3

* *trans*-2-nonenal flavor detection score on a scale of 1 -10

The improved flavor-stability of beer brewed from Line G malt,
as measured by the levels of *trans*-2-nonenal detected in the beer
10 following storage at 30°C from the combined brewing trial data, is
shown to be statistically significant (Table 7).

TABLE 7
TRANS-2-NONENAL IN STORED BEER

fresh	Mean	Stdev	Difference	2-tailed p	Significant (p < 0.05)
reference	0.060	0.012	0.007	0.34	no
line-G	0.053	0.011			

6 weeks, 30 °C	Mean	Stdev	Difference	2-tailed p	Significant (p < 0.05)
reference	0.083	0.013	0.029	0.031	yes
line-G	0.054	0.021			

12 weeks, 30 °C	Mean	Stdev	Difference	2-tailed p	Significant (p < 0.05)
reference	0.114	0.023	0.051	0.003	yes
line-G	0.063	0.020			

Since the natural sulfite levels were low in both brewing trials, the free *trans*-2-nonenal levels in the aged beer would closely reflect the *trans*-2-nonenal potential of the different beers, namely the level of *trans*-2-nonenal adducts present in the fresh beer. Addition of sulfite can temporarily delay the staling process, by complexing free-*trans*-2-nonenal, until sulfite levels are reduced by oxidation due to gaseous exchange through the packaging.

The described brewing trials with low-LOX-1 barley malt provide the first unequivocal evidence that LOX-1 activity in barley during the malting and brewing process is a key determinant of the appearance of the off-flavor compound *trans*-2-nonenal in aged beer.

The above specification includes citations to numerous publications. Each publication is hereby incorporated by reference for all purposes, as if fully set forth.

Applicant's or agent's file reference 11225.11W001	International application No.
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WE CLAIM:

1. A barley plant or portion thereof comprising a mutant LOX-1 protein, the plant or plant portion characterized by a reduction or absence of lipoxygenase activity as compared to a non-mutated control.
2. The barley plant or portion thereof of claim 1, wherein the lipoxygenase activity comprises catalysis of oxidation of free and esterified polyunsaturated fatty acids and polyunsaturated octadecanoic fatty acids to form 9-hydroperoxy fatty acid derivatives.
3. The barley plant or portion thereof of claim 1, wherein the LOX-1 protein is encoded by a mutated *lox-1* nucleic acid sequence having one or more mutation.
4. The barley plant or portion of claim 3, wherein said one or more mutation is induced by chemical mutagenesis or radiation.
5. The barley plant or portion of claim 3, wherein said one or more mutation is induced by site-directed mutagenesis.
6. The barley plant or portion thereof of claim 3, wherein said one or more mutation is positioned within the promoter or transcribed region of the *lox-1* nucleic acid sequence.
7. The barley plant or portion of claim 1, wherein said mutant LOX-1 protein comprises an acidic, basic, or polar amino acid substitution at one or more conserved neutral or hydrophobic amino acid residue lining the substrate cavity of wild-type barley LOX-1.

8. The barley plant or portion of claim 7, wherein said mutant LOX-1 protein comprises the amino acid sequence of SEQ ID NO: 12, wherein Xaa is an acidic, basic, or polar amino acid.
9. The barley plant or portion of claim 8, wherein Xaa is glutamic acid or aspartic acid.
10. The barley plant or portion of claim 9, wherein Xaa is aspartic acid.
11. A transgenic barley plant or portion thereof comprising a heterologous nucleic acid sequence expressing an antisense sequence to at least a portion of a transcribed region of the barley *lox-1* gene, the heterologous sequence operably linked to a gene promoter sequence and a transcription terminator sequence.
12. The transgenic barley plant or portion of claim 11, wherein said gene promoter directs constitutive expression of the antisense sequence.
13. The transgenic barley plant or portion of claim 11, wherein said gene promoter directs tissue-specific or temporally-regulated expression of the operably linked antisense sequence in tissues of the plant.
14. The transgenic barley plant or portion of claim 11, wherein said tissue is developing, germinating, or mature embryonic tissue.
15. The transgenic barley plant or portion of claim 11, wherein said gene promoter sequence is a barley *lox-1* gene promoter or a portion thereof.
16. Grain or plant progeny produced from the barley plant or plant portion of any of claims 1-15.

17. A plant product produced from the barley plant or plant portion of any of claims 1-15 or from the grain or plant progeny of claim 16.
18. The plant product of claim 17, wherein the product is malt.
19. A beverage manufactured using the plant product of any of claims 17-18.
20. Beer manufactured using the plant product of any of claims 17-18.
21. The use of a plant product according to any of claims 17-18 for the preparation of a beverage presenting organoleptic qualities that remain stable over a measured period of time or at elevated storage temperatures as compared to a beverage prepared from a plant product of a non-mutated control.
22. Use of a barley plant or portion thereof, grain or plant progeny, or plant product according to any of claims 1-18 for the manufacture of a beverage.
23. Use of a barley plant or portion thereof, grain or plant progeny, or plant product according to any of claims 1-18 for the manufacture of a malt or beer.
24. Use of a barley plant or portion thereof, grain or plant progeny, or plant product according to any of claims 1-18, for the stabilization of organoleptic properties of a brewed product over a measured period of time as compared to a control brewed product produced using a non-mutated barley plant or portion thereof, grain or plant progeny, or plant product.
25. Use of a barley plant or portion thereof, grain or plant progeny, or plant product according to any of claims 1-18 for the manufacture of a brewed product having reduced levels of free *trans*-2-nonenal over a measured period of time or under conditions of elevated storage temperature, as

compared to a control brewed product produced using a non-mutated barley plant or portion thereof, grain or plant progeny, or plant product.

26. A beverage produced from a barley plant or portion thereof, grain or plant progeny, or plant product according to any of claims 1-18, the beverage having a reduced *trans*-2-nonenal content over a measured period of time or under conditions of elevated storage temperature as, compared to a control beverage produced from a non-mutated barley plant or portion thereof, grain or plant progeny, or plant product.
27. The beverage of claim 26, wherein the beverage is beer.

FIGURE 1

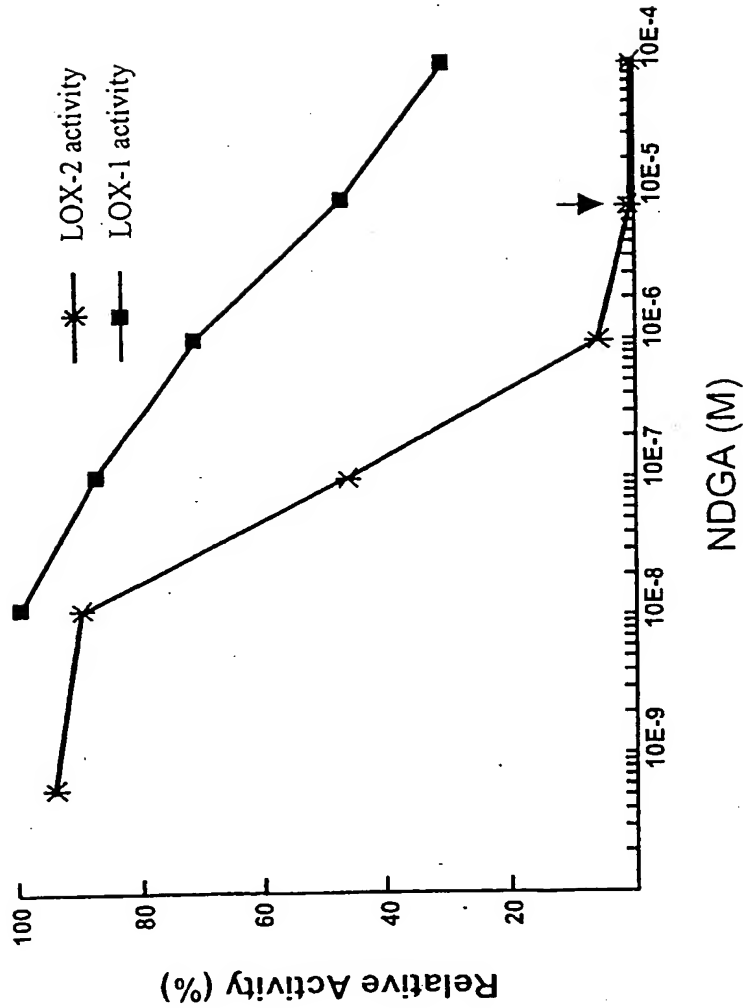


FIGURE 2

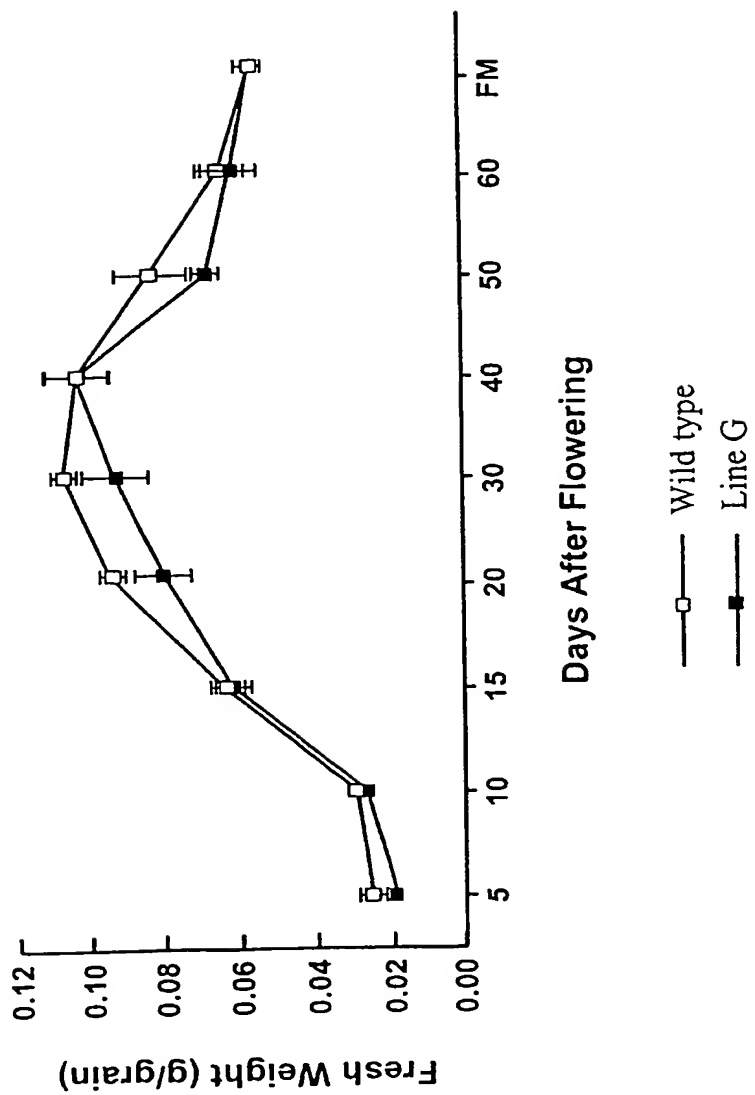


FIGURE 3

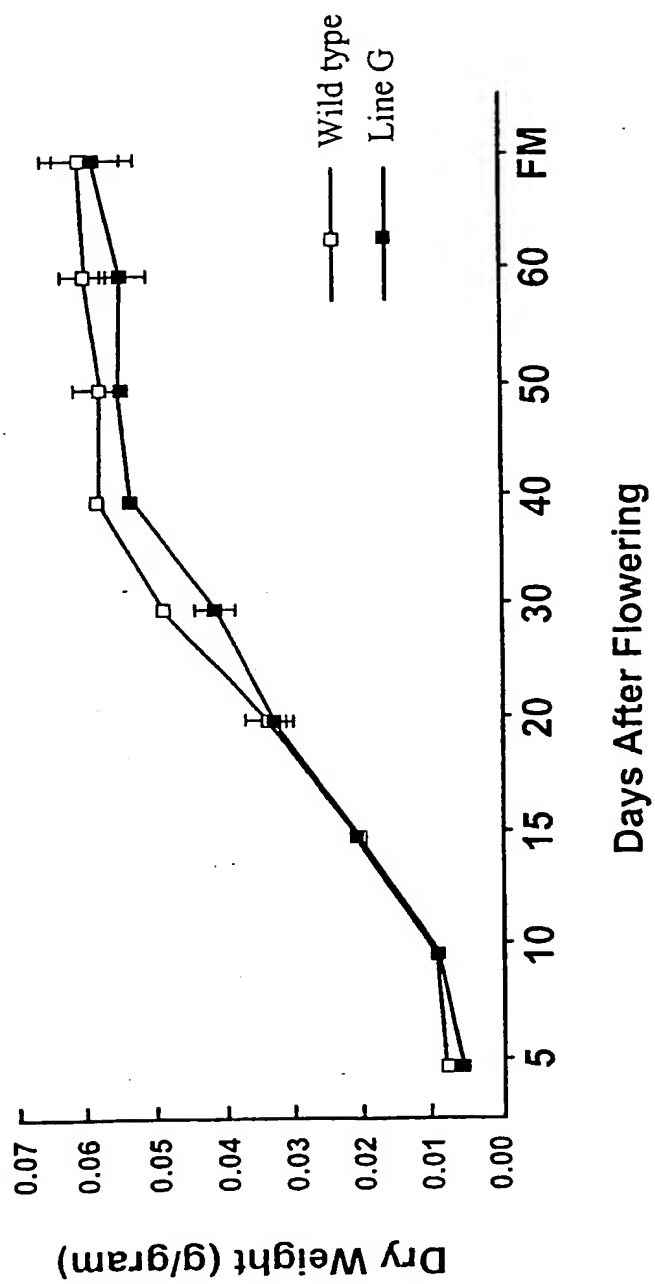


FIGURE 4

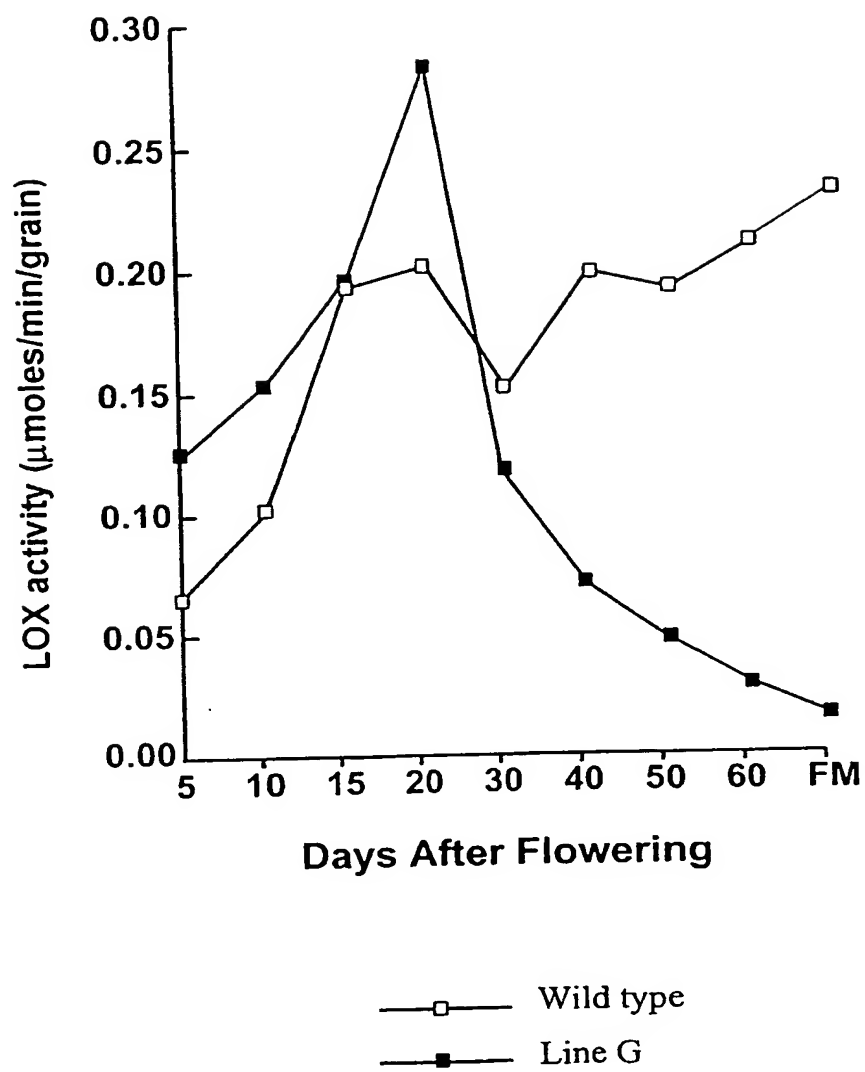


FIGURE 5

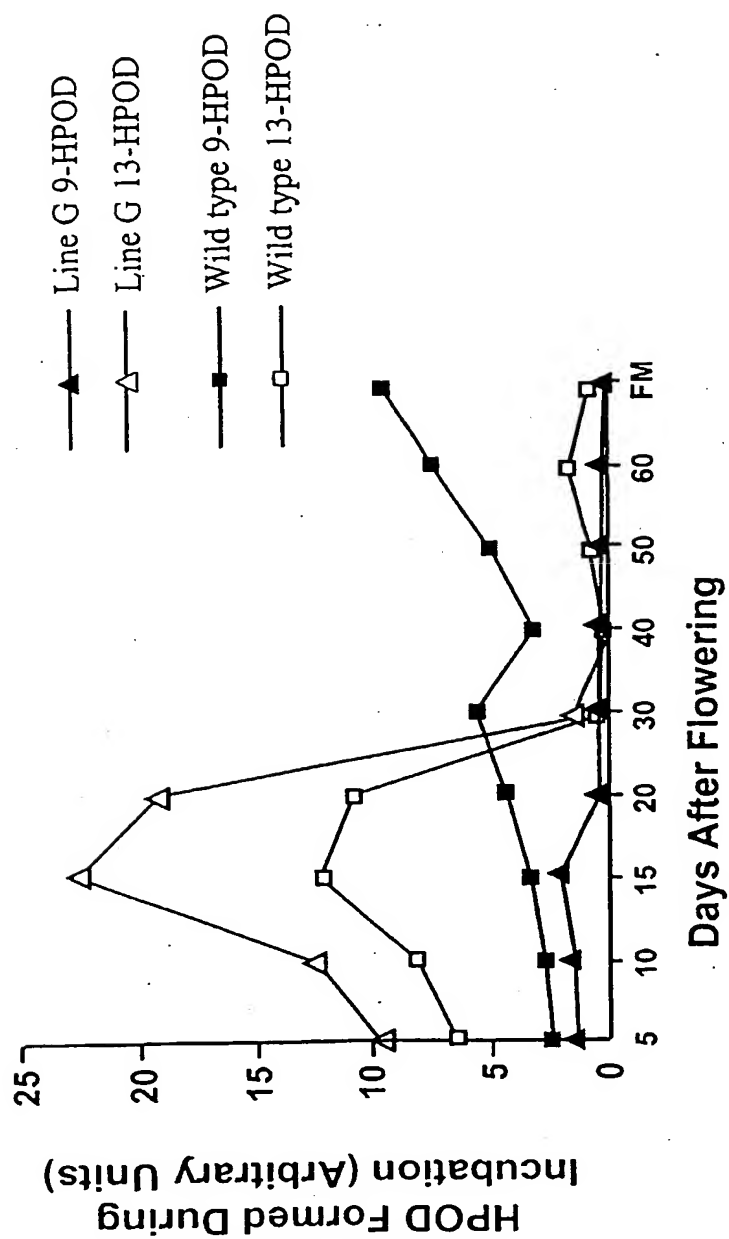


FIGURE 6

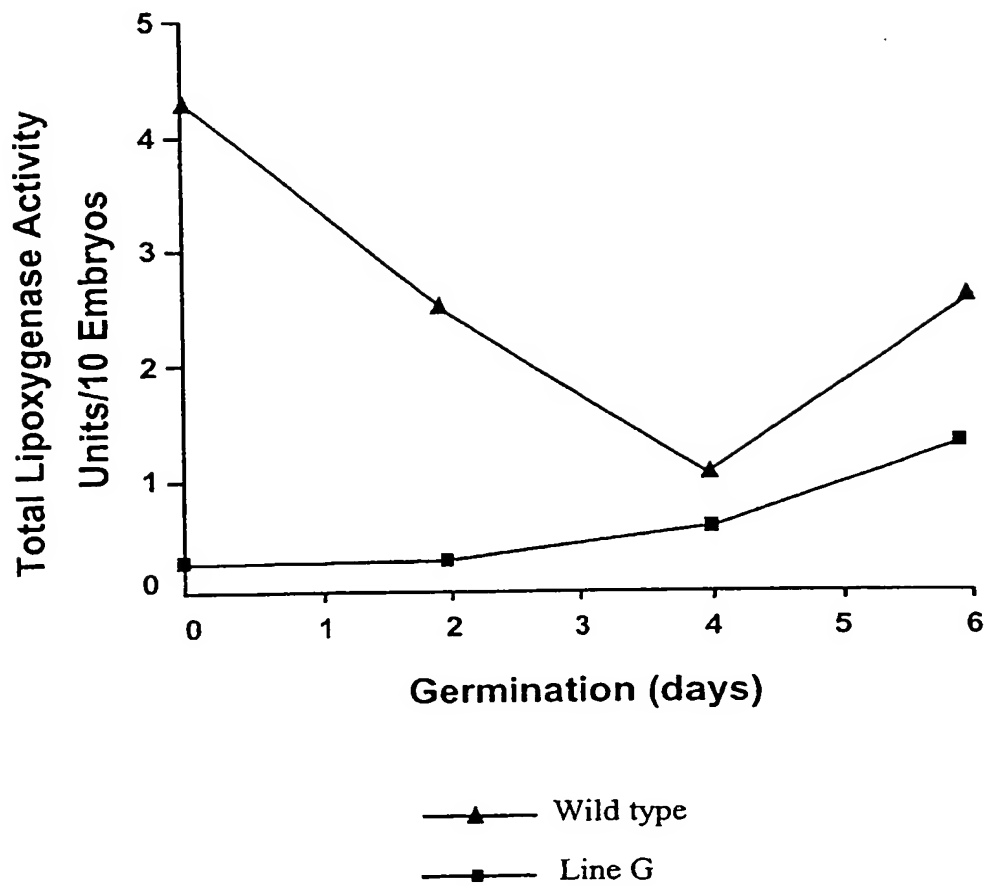


FIGURE 7

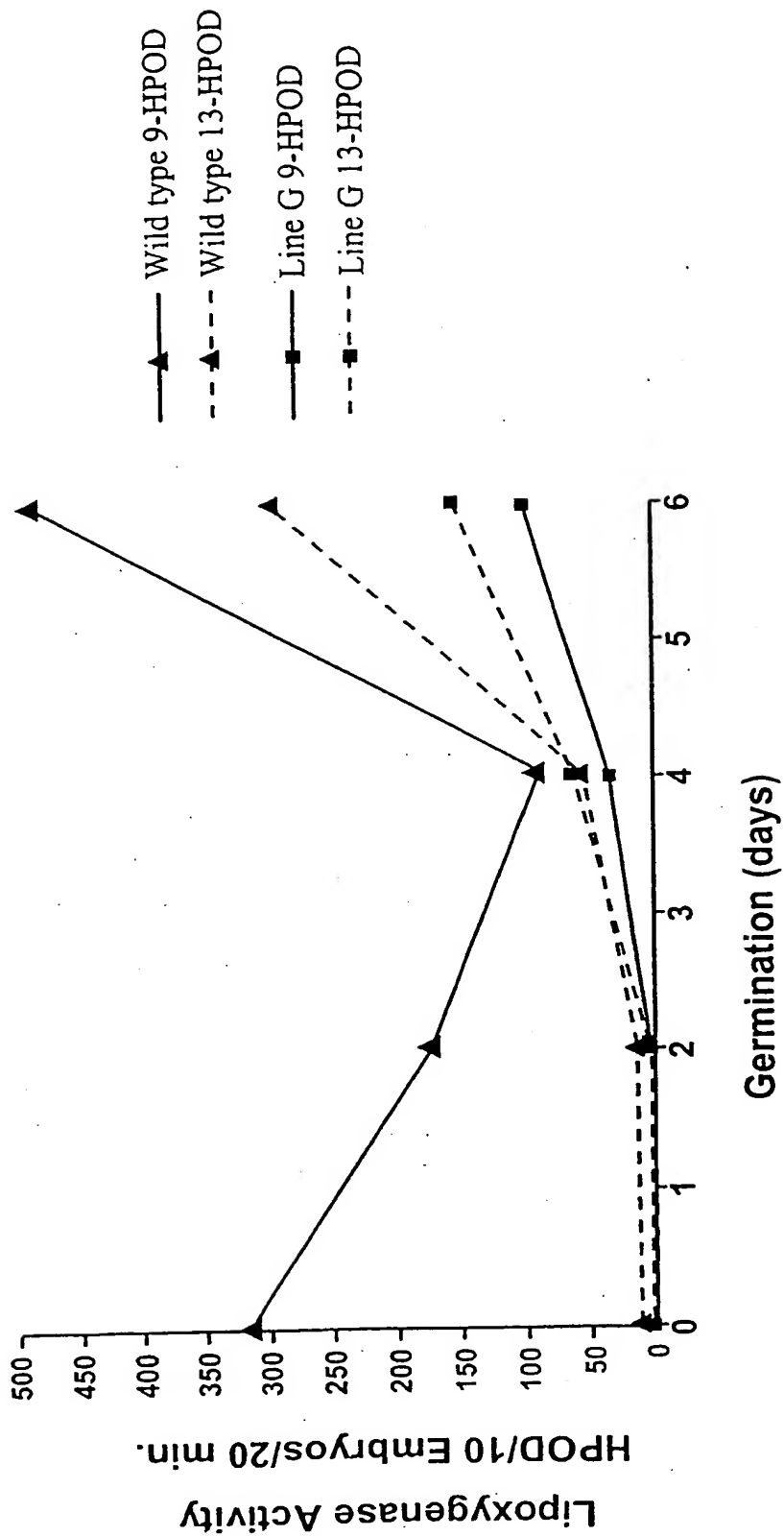


FIGURE 8

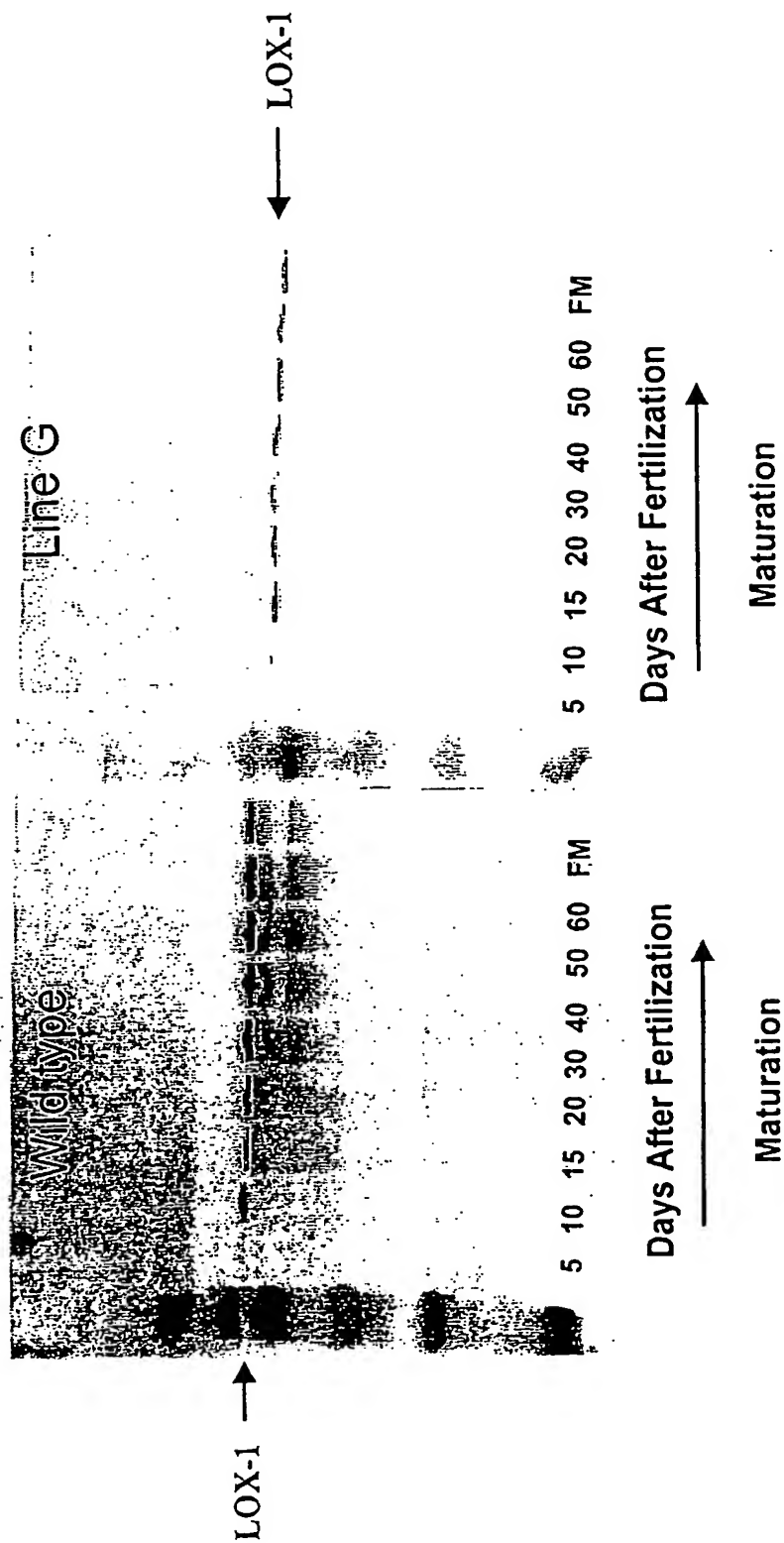


FIGURE 9

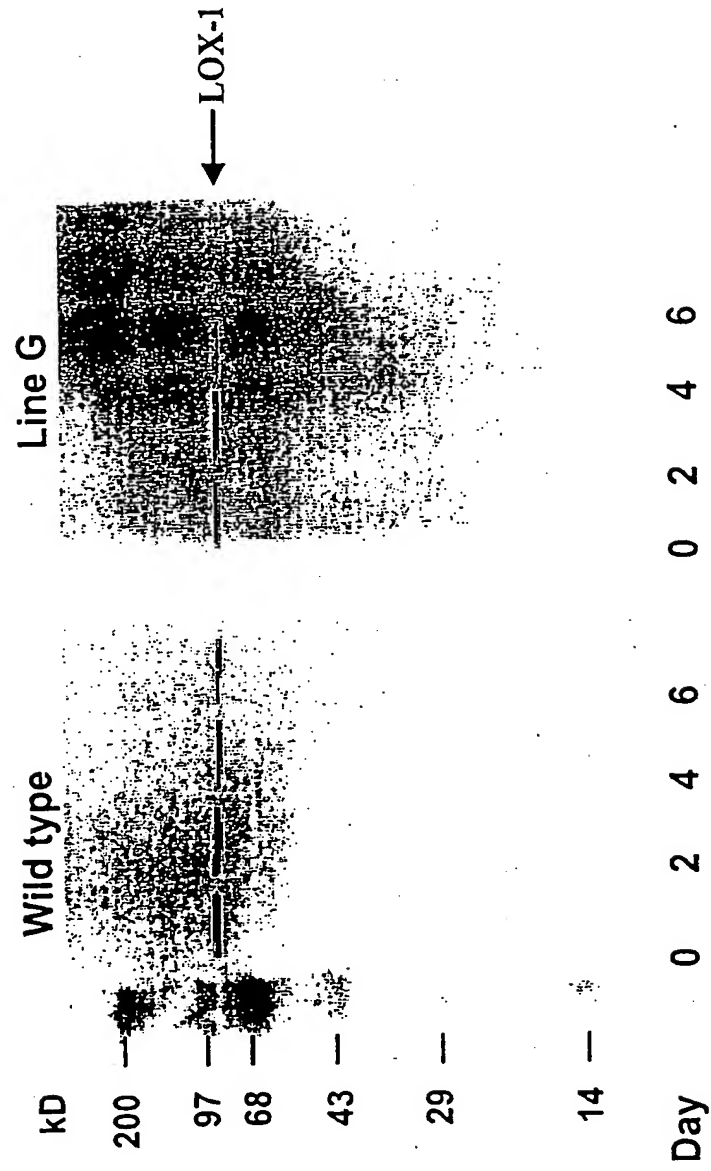


FIGURE 10

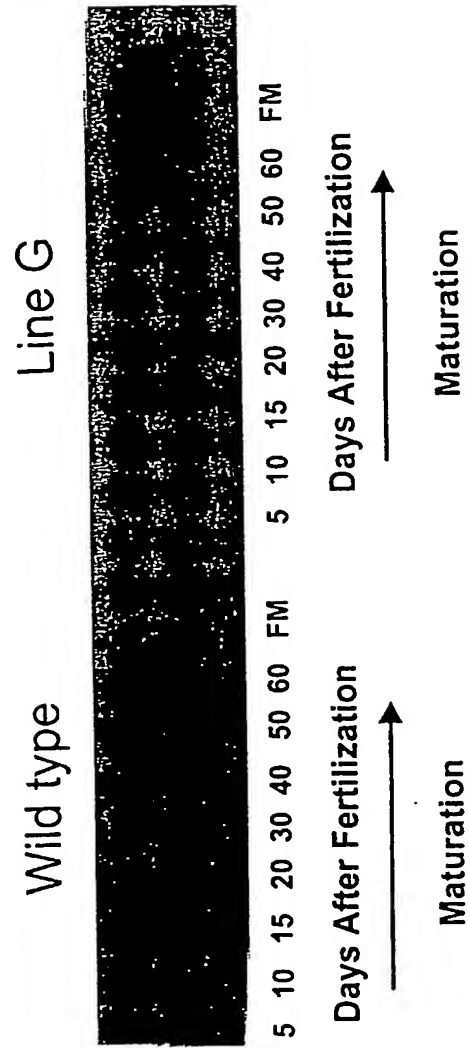


FIGURE 11

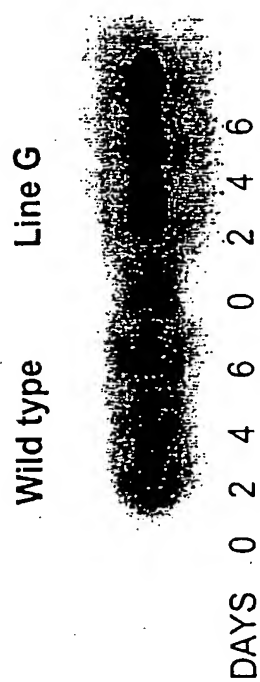


FIGURE 12A

(-288) Promoter

LG CAGCCCCATGCATGCACATGCACATGCACATGCAGTGAGCCAGCAACCGCTCGATGGCGGATCAGCCCGTCACG -208

WT CAGCCCCATGCATGCACATGCACATGCACATGCACATGCAGTGAGCCAGCAACCGCTCGATGGCGGATCAGCCCGTCACG

LG GGACCGGAGCGGCCATGCGAAGCAGCAGGAGGGACGTCACCGTCCGCGCGCAGCACGCTGGAGAGCACGTCGCCGTCGG -128

WT GGACCGGAGCGGCCATGCGAAGCAGCAGGAGGGCACGTCACCGTCCGCGCGCAGCACGCTGGAGAGCACGTCGCCGTCGG

LG ATCCATCTCTCCAAAGCCGAGCGCCACACACCACCGGACCGGACCGGCCTATAAAATGCCCGGACCGAGCTGCAA -48

WT ATCCATCTCTCCAAAGCCGAGCGCCACACACCACCGGACCGGACCGGCCTATAAAATGCCCGGACCGAGCTGCAA

Transcription start site (+1)

LG GCAGCTCCTCACACACACTCAGCGCAACACACATCCATCTTCACTGAAAAGTGAAAAACAGTGTGCTGGTGCCATTGGTTG 32

WT GCAGCTCCTCACACACACTCAGCGCAACACACATCCATCTTCACTGAAAAGTGAAAAACAGTGTGCTGGTGCCATTGGTTG

Translation start site (+69) →

LG GAGCAGTGAAAGCGAGGAGGAGGCCCAAGAACAAAGATGCTGTGGAGGGCTGATCGACACCCCTACGGGGCGGAACAA 112

WT GAGCAGTGAAAGCGAGGAGGAGGCCCAAGAACAAAGATGCTGTGGAGGGCTGATCGACACCCCTACCGGGGGCGGAACAA

LG GAGCGCCCGGCTCAAGGGCACGGTGGTGCTCATGCGCAAGAACGTGCTGACCTCAACGACTTCGGCGCCACCATCATCG 192

WT GAGCGCCCGGCTCAAGGGCACGGTGGTGCTCATGCGCAAGAACGTGCTGACCTCAACGACTTCGGCGCCACCATCATCG

(Silent mutation)*

LG ACGGCATCGGCGAGTTCTCTGGCAAGGGTGTACCTGCCAGCTTATCAGCTCCACCGCGTCCGACCAAGGTAATCACTAC 272

WT ACGGCATCGGCGAGTTCTCTGGCAAGGGGTCACCTGCCAGCTTATCAGCTCCACCGCGTCCGACCAAGGTAATCACTAC

LG CCTCCTCCGGCCTTCTCCTGTGTTTACAAGATATAGTATTCTTTCGTGTGGCGCGGCCCATGGATGGATGGATGTGT 352

WT CCTCCTCCGGCCTTCTCCTGTGTTTACAAGATATAGTATTCTTTCGTGTGGCGCGGCCCATGGATGGATGGATGTGT

FIGURE 12B

LG CTGGATCGGCTAAAGAAGATAGGATAGCTAGCCCTGGCCGGTCGTCTTTACCTGAGCATGGGCATATGCCATCGAAAAAA 432
 WT CTGGATCGGCTAAAGAAGATAGGATAGCTAGCCCTGGCCGGTCGTCTTTACCTGAGCATGGGCATATGCCATCGAAAAAA

 LG GAGACAAACAGCATGCATGCTGCTGGCGGCCACAGACCCAGAGCACCAGATGCTCGAGACAAAGCAACACAACAGC 512
 WT GAGACAAACAGCATGCATGCTGCTGGCGGCCACAGACCCAGAGCACCAGATGCTCGAGACAAAGCAACACAACAGC

 LG AAGGACGACACGTCATAAAGCAACACAAAGCAAGGACGGCACGTCATAAAGCAACACAAACCTAAA CTAAAGCACAAAGA 592
 WT AAGGACGACACGTCATAAAGCAACACAAAGCAAGGACGGCACGTCATAAAGCAACACAAACCTAAA CTAAAGCACAAAGA

 LG CGTAAGAGCAAGCACACAATCAGCAGGCTATAAACAGTTGTCTATCAAAAAACAACGCTGGAAGAGAGAGAGAAAGGAA 672
 WT CGTAAGAGCAAGCACACAATCAGCAGGCTATAAACAGTTGTCTATCAAAAAACAACGCTGGAAGAGAGAGAGAAAGGAA

 LG GTAGTAGCCATGAAAAATTAAATCACCGGGCGTTGCTCTTTGCCCAACAATTAATCAAGCAGGGTACGTGGCATGTATAG 752
 WT GTAGTAGCCATGAAAAATTAAATCACCGGGCGTTGCTCTTTGCCCAACAATTAATCAAGCAGGGTACGTGGCATGTATAG

 LG TTCTTTGTAAGTAACTAAGCATGTGATATGAGAAAGGTACGTGGTGGTGACAGACAACGGCGGTGCGGGGAAGGTGGGCGCG 832
 WT TTCTTTGTAAGTAACTAAGCATGTGATATGAGAAAGGTACGTGGTGGTGACAGACAACGGCGGTGCGGGGAAGGTGGGCGCG

 LG GAGGCGGAGCTGGAGCAGTGGGTGACGAGCCTGCCGTGCTGACGACGGGGAGTCCAAAGTTCGGCCTCACCTTCGACTG 912
 WT GAGGCGGAGCTGGAGCAGTGGGTGACGAGCCTGCCGTGCTGACGACGGGGAGTCCAAAGTTCGGCCTCACCTTCGACTG

 LG GGAGGTGGAGAAAGCTCGGGGTGCCGGGGCCCATCGTCGTCAACAACACTACCACAGCTCCGAGTTCCTGCTTAAACCATCA 992
 WT GGAGGTGGAGAAAGCTCGGGGTGCCGGGGCCCATCGTCGTCAACAACACTACCACAGCTCCGAGTTCCTGCTTAAACCATCA

 LG CCTCCACGACGTCCCCCGCGCAGCGGCAACCTCACCTTCGTGCGCAACTCATGGATCTACCCCGCGGCCAACTACCGA 1072
 WT CCTCCACGACGTCCCCCGCGCAGCGGCAACCTCACCTTCGTGCGCAACTCATGGATCTACCCCGCGGCCAACTACCGA

FIGURE 12C

LG TACAGCCGCGTCTTCTTCGCCCAACGACGTGCGTGGATTTTCCCTCTACTTTCCCTCTCCTTTTCATTTTCACCGCCTTCGTCA 1152
 WT TACAGCCGCGTCTTCTTCGCCCAACGACGTGCGTGGATTTTCCCTCTACTTTCCCTCTCCTTTTCATTTTCACCGCCTTCGTCA

 LG TTCAATGGTCGATCATTAAGTCTTGCCAGGACAATAGATGATGAGCTAGGAGTGGTTACCACTTAGCAGTACGTACATTAT 1232
 WT TTCAATGGTCGATCATTAAGTCTTGCCAGGACAATAGATGATGAGCTAGGAGTGGTTACCACTTAGCAGTACGTACATTAT

 LG TTATTCCCGTGTGGTAGAAAAAGGATATGGTTTGGTGCAGATCGACACAAGATTGAATGAAAGTTGCACCCGTGGCACCCGTG 1312
 WT TTATTCCCGTGTGGTAGAAAAAGGATATGGTTTGGTGCAGATCGACACAAGATTGAATGAAAGTTGCACCCGTGGCACCCGTG

 LG GCAGCGGTAGGTGAAAAATAACTGTGTCACGGATCCACCCACATGATTGTTTTCATGAAATAAACTTTTAAAGGATGTGT 1392
 WT GCAGCGGTAGGTGAAAAATAACTGTGTCACGGATCCACCCACATGATTGTTTTCATGAAATAAACTTTTAAAGGATGTGT

 LG CTAGCCACATCTAGATGCATGTCACATAAATTATTGCATACCAAACCGATTAAATTAAGCATAAAAAGAAAAAAA 1472
 WT CTAGCCACATCTAGATGCATGTCACATAAATTATTGCATACCAAACCGATTAAATTAAGCATAAAAAGAAAAAAA

 LG TACTCACATATCTCGACGTAAGATCAATGATATAGTATTTAGATATGCAATATTTATCTTACATCTAAACCTTTCTTTCAT 1552
 WT TACTCACATATCTCGACGTAAGATCAATGATATAGTATTTAGATATGCAATATTTATCTTACATCTAAACCTTTCTTTCAT

 LG TCTTAAATATAAGACATTTTGTAAGATTTCACTATGGACAACATACGAAACAAAATCAGTGGATCTCTCTATGCATTTCATT 1632
 WT TCTTAAATATAAGACATTTTGTAAGATTTCACTATGGACAACATACGAAACAAAATCAGTGGATCTCTCTATGCATTTCATT

 LG ATGTAGTCTATAATAAAAAATCTTTAAAGATCGTATATTTTGCAACGGAGGGAGTAAAAACATAACTTTTTTAATAGTAATGT 1712
 WT ATGTAGTCTATAATAAAAAATCTTTAAAGATCGTATATTTTGCAACGGAGGGAGTAAAAACATAACTTTTTTAATAGTAATGT

FIGURE 12D

LG TGCACGGCTCCACACTCGCAGACGTACTGTGCCGAGCCAGATGCCGGGGGGCTGAAGCCGTACCGCGACGACGAGCTCCG 1792
 WT TGCACGGCTCCACACTCGCAGACGTACTGTGCCGAGCCAGATGCCGGGGGGCTGAAGCCGTACCGCGACGACGAGCTCCG

LG GAACCTGCTGGCGACGACGAGAGGGCCCGTACCAGGAGCAGACCGCATCTACCGCTACGACGTCTACAACGACCTCG 1872
 WT GAACCTGCTGGCGACGACGAGCCAGGGCCCGTACCAGGAGCAGACCGCATCTACCGCTACGACGTCTACAACGACCTCG

LG GCGAGGGCGGCCCCATCCTCGGGGGCAACTCCGACCAACCCTTACCCGGCCCGCGCGCCGACGAGCGCAAGCCCCAACGCC 1952
 WT GCGAGGGCGGCCCCATCCTCGGGGGCAACTCCGACCAACCCTTACCCGGCCCGCGCGCCGACGAGCGCAAGCCCCAACGCC

LG AGCGACCCGAGCCTGGAGAGCCGGCTGTGCTGCTGGAGCAGATCTACGTGCCGGGGACGAGAAAGTTCGGGCCACCTCAA 2032
 WT AGCGACCCGAGCCTGGAGAGCCGGCTGTGCTGCTGGAGCAGATCTACGTGCCGGGGACGAGAAAGTTCGGGCCACCTCAA

LG GACGTCCGACTTCCCTGGGCTACTCCATCAAGGCCATCAGCAGGGCATCCTGCCGGCCGTGCGCACCTACGTGGACACCA 2112
 WT GACGTCCGACTTCCCTGGGCTACTCCATCAAGGCCATCAGCAGGGCATCCTGCCGGCCGTGCGCACCTACGTGGACACCA

LG CCCCCGGGAGTTCGACTCCTTCCAGGACATCAACCTCTATGAGGGCGGCATCAAGCTGCCCCAAGGTGGCCGCCCTG 2192
 WT CCCCCGGGAGTTCGACTCCTTCCAGGACATCAACCTCTATGAGGGCGGCATCAAGCTGCCCCAAGGTGGCCGCCCTG

LG GAGGAGTCCGTAAGCAGTTCCCGCTCCAGCTCATCAAGGACCTCCTCCCCGTGCGGGCGGACTCCCTGCTTAAGCTCCC 2272
 WT GAGGAGTCCGTAAGCAGTTCCCGCTCCAGCTCATCAAGGACCTCCTCCCCGTGCGGGCGGACTCCCTGCTTAAGCTCCC

LG CGTGCCCCACATCATCCAGGAGAACAAAGCAGGCGTGGAGACCGACGAGGAGTTCGCACGGGAGGTGCTCGCCGACGTCA 2352
 WT CGTGCCCCACATCATCCAGGAGAACAAAGCAGGCGTGGAGACCGACGAGGAGTTCGCACGGGAGGTGCTCGCCGGCGTCA

(Mutant site) *

FIGURE 12E

LG ACCCGGTCAATGATCAGCGGTCTCAGCGTGAGTCAGCGATTATTTGTTTCAATGTTGTGTATGGTGTCCATGGTGAGAAAG 2432
 WT ACCCGGTCAATGATCAGCGGTCTCAGCGTGAGTCAGCGATTATTTGTTTCAATGTTGTGTATGGTGTCCATGGTGAGAAAG

 LG TGCAGATCTTGATTTGCGTTGGTTCGCATGCACCGATGCTGCATGCATGCAGGAGTTCCCGCCAAAAGTAGTCTGGACC 2512
 WT TGCAGATCTTGATTTGCGTTGGTTCGCATGCACCGATGCTGCATGCATGCAGGAGTTCCCGCCAAAAGTAGTCTGGACC

 LG CTAGCAAGTTTGGTGACCAACACAGCACCATCAGGCGGAGCACATAGAGAAGAACCTCGAGGGCCTCACGGTGCAGCAG 2592
 WT CTAGCAAGTTTGGTGACCAACACAGCACCATCAGGCGGAGCACATAGAGAAGAACCTCGAGGGCCTCACGGTGCAGCAG

 LG GTAATTGGTCCAAGCCATCGACATCAACTATGATTTACCTAGGAGTAATTGGTAGCTGTAGATAAATTTGGCTTCGTTGCA 2672
 WT GTAATTGGTCCAAGCCATCGACATCAACTATGATTTACCTAGGAGTAATTGGTAGCTGTAGATAAATTTGGCTTCGTTGCA

 LG ATTAATTTGATGCTGGCCGATCAAGTGATCGTATTGGGTTTGAAATTTGCAGGCGCTGGAAGCAACAGGCTGTACATCC 2752
 WT ATTAATTTGATGCTGGCCGATCAAGTGATCGTATTGGGTTTGAAATTTGCAGGCGCTGGAAGCAACAGGCTGTACATCC

 LG TTGATCACCATGACCGGTTTCATGCCGTTCCCTGATCGACGTCAACAACTGCCCGGCAACTTCATCTACGCCACGAGGACC 2832
 WT TTGATCACCATGACCGGTTTCATGCCGTTCCCTGATCGACGTCAACAACTGCCCGGCAACTTCATCTACGCCACGAGGACC

 LG CTCCTTCTCCTGCGCGGACGGCAGGCTCACGCCGCTCGCCATCGAGCTGAGCGAGCCCCATCATCCAGGGCGGCCTTAC 2912
 WT CTCCTTCTCCTGCGCGGACGGCAGGCTCACGCCGCTCGCCATCGAGCTGAGCGAGCCCCATCATCCAGGGCGGCCTTAC

 LG CACGGCCAAAGAGCAAGGTTTACACGCCGGTGCCCAAGGCTCCGTGCAAGGCTGGGTGTGGGAGCTCGCCAAAGGCCTACG 2992
 WT CACGGCCAAAGAGCAAGGTTTACACGCCGGTGCCCAAGGCTCCGTGCAAGGCTGGGTGTGGGAGCTCGCCAAAGGCCTACG

 LG TCGCCGTCAATGACTCCGGGTGGCACCAGCTCGTCAGCCACTGGTACGTTCTCCACGGTCGATGTGATTCAGTCAGTCGA 3072
 WT TCGCCGTCAATGACTCCGGGTGGCACCAGCTCGTCAGCCACTGGTACGTTCTCCACGGTCGATGTGATTCAGTCAGTCGA

FIGURE 12F

LG TGCACAACAACACTGATCGAAATATGATTGATTGAAACGCGCAGGCTGAACACTCACGCGGTGATGGAGCCGTTTCGTGATCT 3152
 WT TGCACAACAACACTGATCGAAATATGATTGATTGAAACGCGCAGGCTGAACACTCACGCGGTGATGGAGCCGTTTCGTGATCT

LG CGACGAACCGGCACCTTAGCGTGACGCACCCGGTGCAAGCTGCTGAGCCCGCACTACCGCGACACCATGACCATCAAC 3232
 WT CGACGAACCGGCACCTTAGCGTGACGCACCCGGTGCAAGCTGCTGAGCCCGCACTACCGCGACACCATGACCATCAAC

LG GCGCTGGCGCGGCAGACGCTCATCAACGCGCGGGCATCTTCGAGATGACGGTGTTCGCCGGCAAGTTCGCGTTGGGGAT 3312
 WT GCGCTGGCGCGGCAGACGCTCATCAACGCGCGGGCATCTTCGAGATGACGGTGTTCGCCGGCAAGTTCGCGTTGGGGAT

LG GTCGCGCGGTGGTGACAAGGACTGGAAGTTCAACGAGCAGGACTGCCGGACGATCTCATCAAGAGGTACGTACCTGGTA 3392
 WT GTCGCGCGGTGGTGACAAGGACTGGAAGTTCAACGAGCAGGACTGCCGGACGATCTCATCAAGAGGTACGTACCTGGTA

LG AATGTTATGAATGTGTAAACAATAATTGGGCGTCTCGCTCACTGACAGGAACGTGGTAAAAAATAATGCAGGGCATGGCCG 3472
 WT AATGTTATGAATGTGTAAACAATAATTGGGCGTCTCGCTCACTGACAGGAACGTGGTAAAAAATAATGCAGGGCATGGCCG

LG TGGAGGACCCGTCGAGCCCGTACAAGGTGCGGTTGCTGCTGTCGGAATAACCGTACCGCGCGGACGGGCTGGCGATCTGG 3552
 WT TGGAGGACCCGTCGAGCCCGTACAAGGTGCGGTTGCTGCTGTCGGAATAACCGTACCGCGCGGACGGGCTGGCGATCTGG

LG CACGCCATTGAGCAGTACGTGAGCGAGTACCTGGCCATCTACTACCCGAACGACCGCGTGCTGCAGGGCGGATACGGAGGT 3632
 WT CACGCCATTGAGCAGTACGTGAGCGAGTACCTGGCCATCTACTACCCGAACGACCGCGTGCTGCAGGGCGGATACGGAGGT

LG GCAGGCGTGGTGAAGGAGACGCGCGAGGTGGGACGGCGACCTCAAGACGCCCCCATGGTGGCCCCAAGATGCAAAAGTG 3712
 WT GCAGGCGTGGTGAAGGAGACGCGCGAGGTGGGACGGCGACCTCAAGACGCCCCCATGGTGGCCCCAAGATGCAAAAGTG

FIGURE 12G

LG TGCCGGAGCTGGCCAAAGCGTGCACCAACCATCATCTGGATCGGGTCGGCGCTGCATCGGCAGTCAACTTCGGGCAGTAC 3792
WT TGCCGGAGCTGGCCAAAGCGTGCACCAACCATCATCTGGATCGGGTCGGCGCTGCATCGGCAGTCAACTTCGGGCAGTAC

LG CCCTACGCGGGGTTCTCCGAAACCGGCCGACGGTGAGCCGGCGCGCATGCCGGAGCCGGCACGGAGGAGTAGCGCGGA 3872
WT CCCTACGCGGGGTTCTCCGAAACCGGCCGACGGTGAGCCGGCGCGCATGCCGGAGCCGGCACGGAGGAGTAGCGCGGA

LG GCTGAGCGGACCCGGAGCGGGCCTTCATCCACACCATCACAGAGCCAGATCCAGACCATCATCGGCGTGTCTGCTGTGG 3952
WT GCTGAGCGCGACCCGGAGCGGGCCTTCATCCACACCATCACAGAGCCAGATCCAGACCATCATCGGCGTGTCTGCTGTGG

LG AGGTGCTGTGAAAGCACTCCTCCGACGAGCTGTACCTCGGGCAGCGGGACACGCCGAGTGGACCTCGGACCCAAAGGCC 4032
WT AGGTGCTGTGAAAGCACTCCTCCGACGAGCTGTACCTCGGGCAGCGGGACACGCCGAGTGGACCTCGGACCCAAAGGCC

LG CTGGAGGTGTTCAAGCGGTTACAGCGACCGGCTGGTGAGATCGAGAGCAAGGTGGTGGGCATGAACCATGACCCCGAGCT 4112
WT CTGGAGGTGTTCAAGCGGTTACAGCGACCGGCTGGTGAGATCGAGAGCAAGGTGGTGGGCATGAACCATGACCCCGAGCT

LG CAAGAACCGCAACGGCCCGGCTAAGTTTCCCTACATGCTGCTCTACCCCAACACCTCCGACCAAGGGCGCGCTGCCG 4192
WT CAAGAACCGCAACGGCCCGGCTAAGTTTCCCTACATGCTGCTCTACCCCAACACCTCCGACCAAGGGCGCGCTGCCG

translation stop (4229)

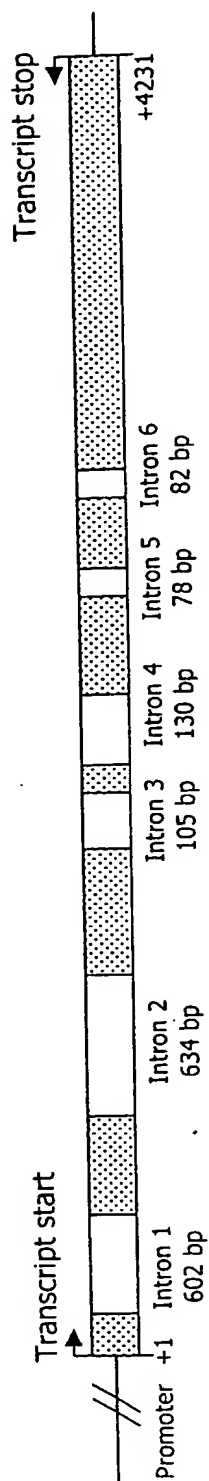
LG GGCTTACCGCCAAAGGGCATCCCCAACAGCATCTCCATCTAATCTAAGCCATCGGCACCATGGATGAATAAAGGGCGTTC 4272
WT GGCTTACCGCCAAAGGGCATCCCCAACAGCATCTCCATCTAATCTAAGCCATCGGCACCATGGATGAATAAAGGGCGTTC

LG GCCACGTACGAAACTTGTGAGAGATTGGTGTAGTGTGTGTGTGACAGTACTATGTCAGCAGTTGCTCTTTAAGCCGA 4352
WT GCCACGTACGAAACTTGTGAGAGATTGGTGTAGTGTGTGTGTGACAGTACTATGTCAGCAGTTGCTCTTTAAGCCGA

LG ATAAATAAAGCAGATTGTGCTTCC 4375
WT ATAAATAAAGCAGATTGTGCTTCC

FIGURE 13

Wild-type *LOX-1* gene structure:



Line G *lox-1* gene structure:

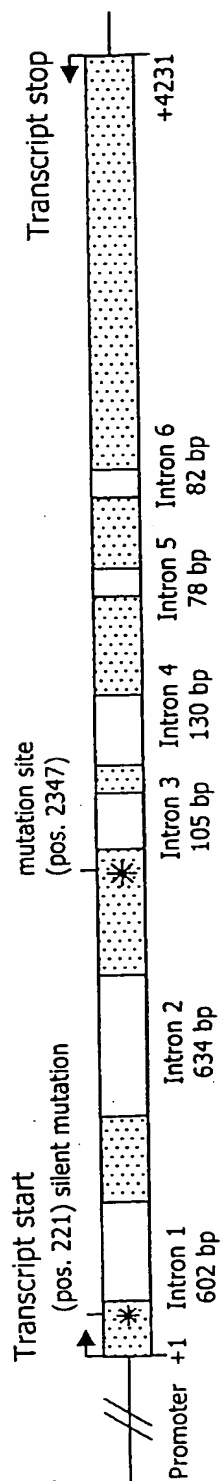


FIGURE 14

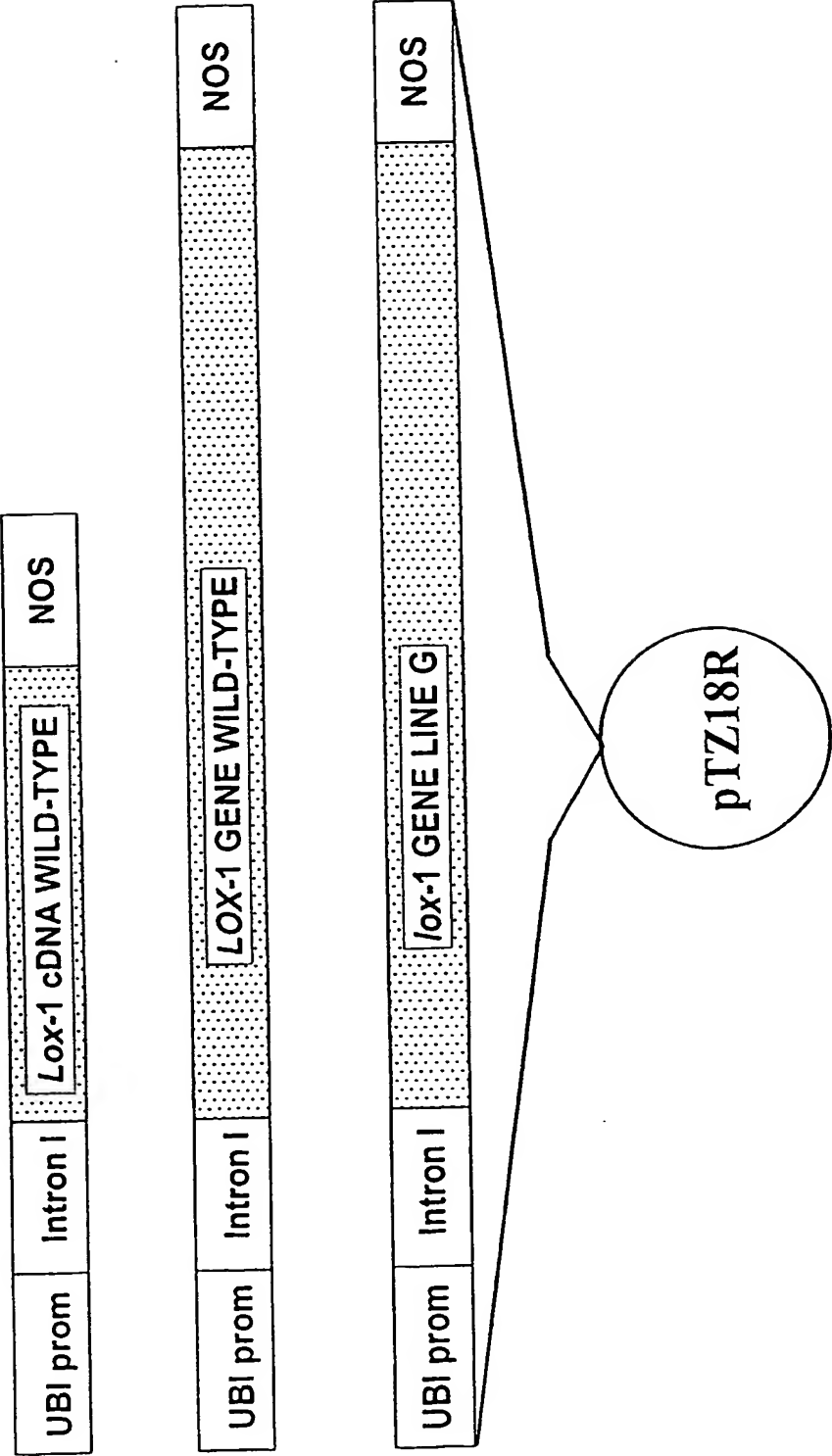


FIGURE 15

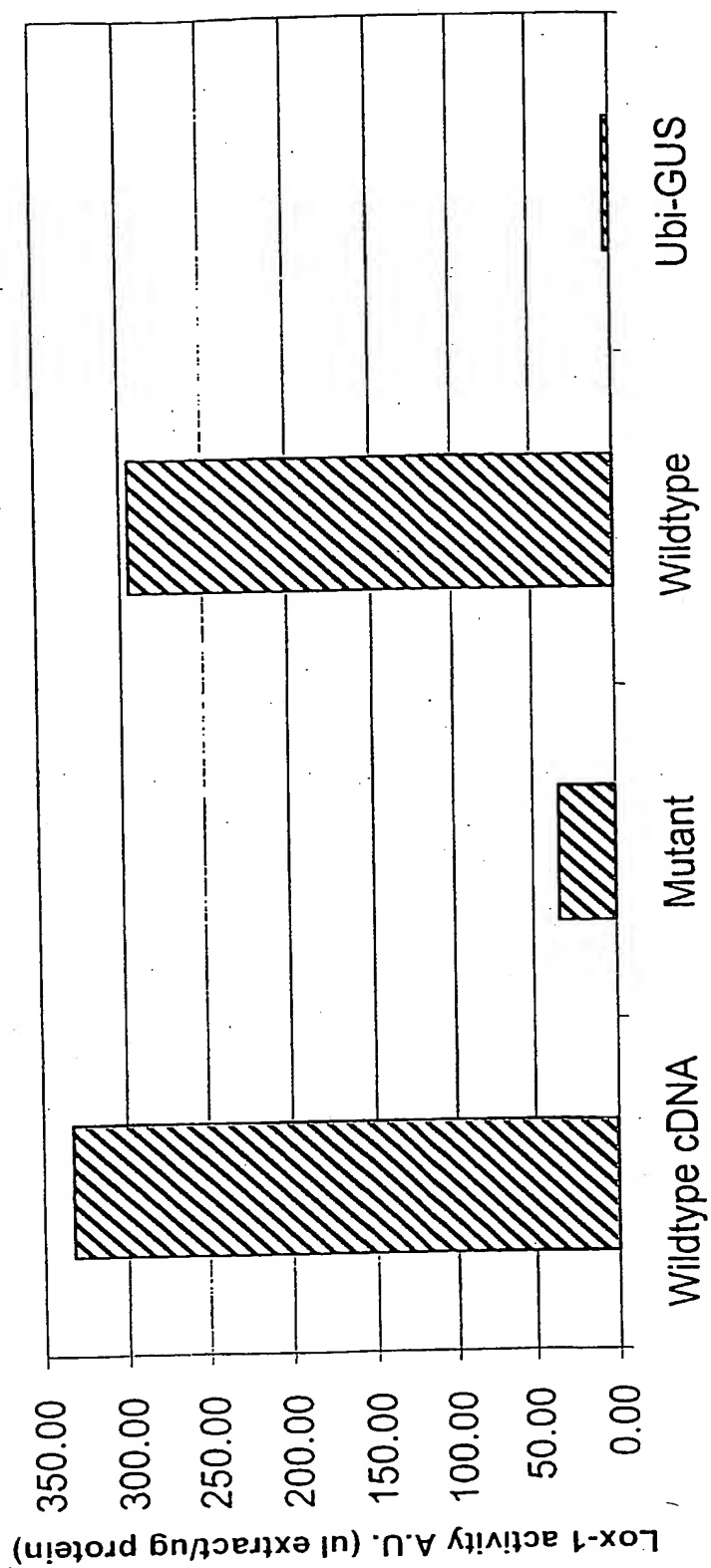


FIGURE 16

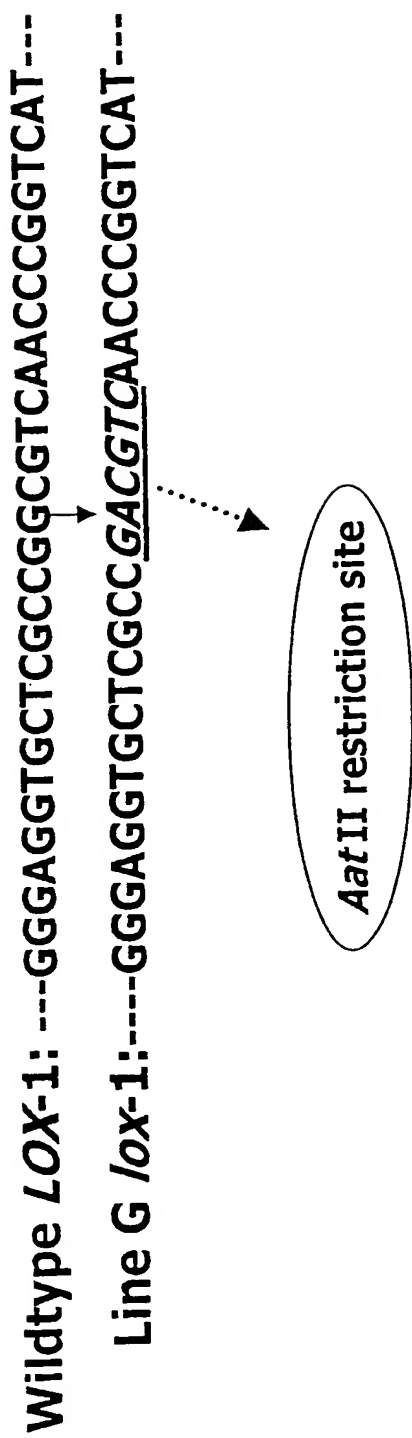


FIGURE 17

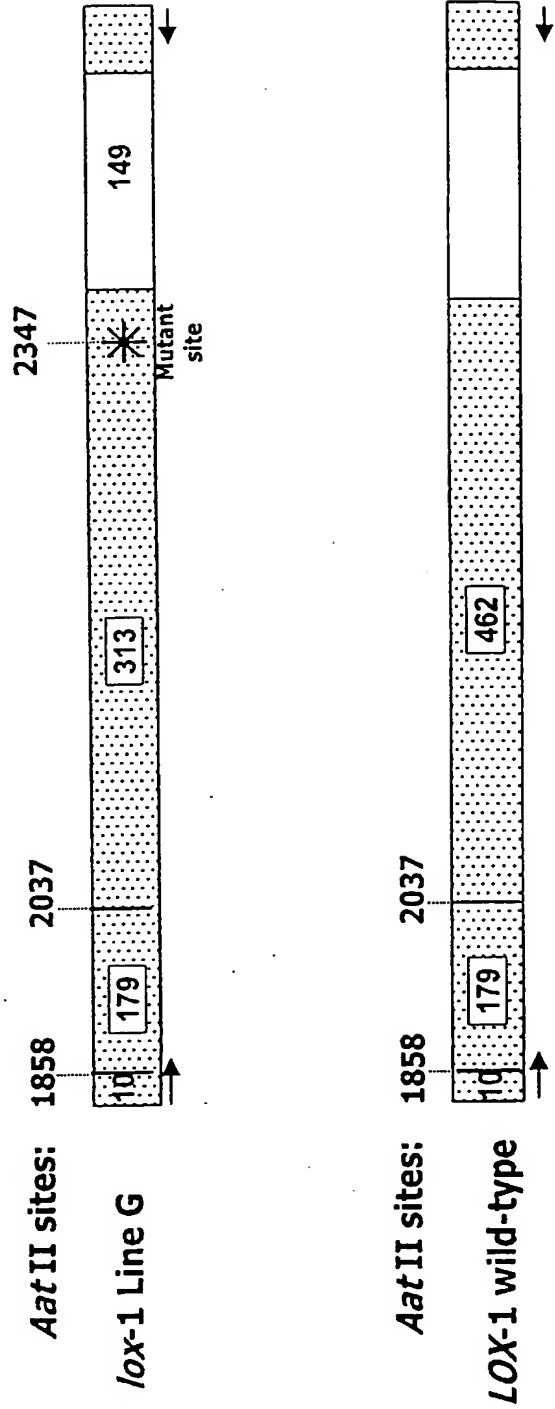


FIGURE 18

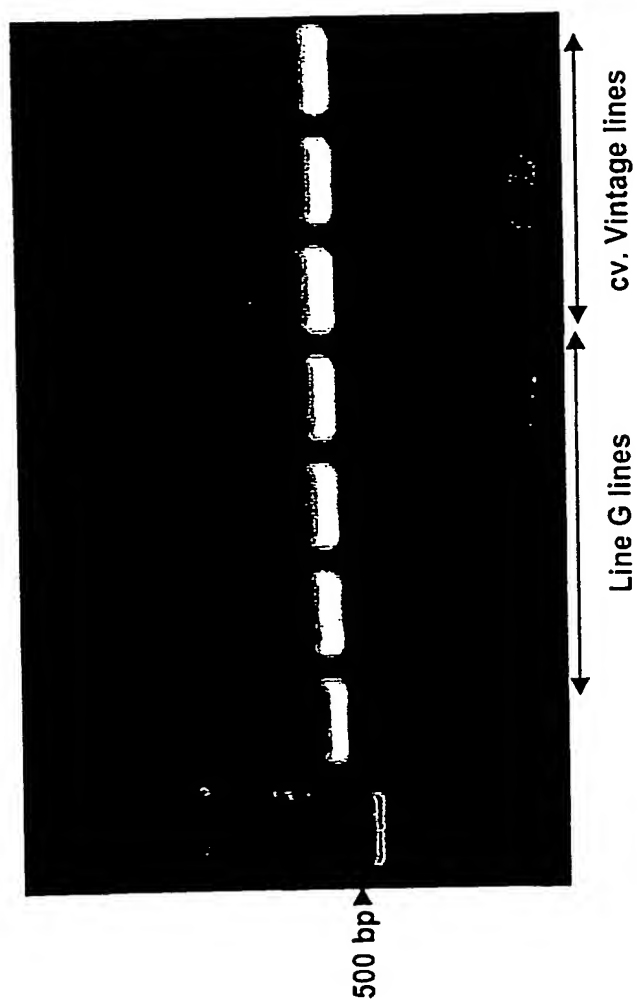


FIGURE 21

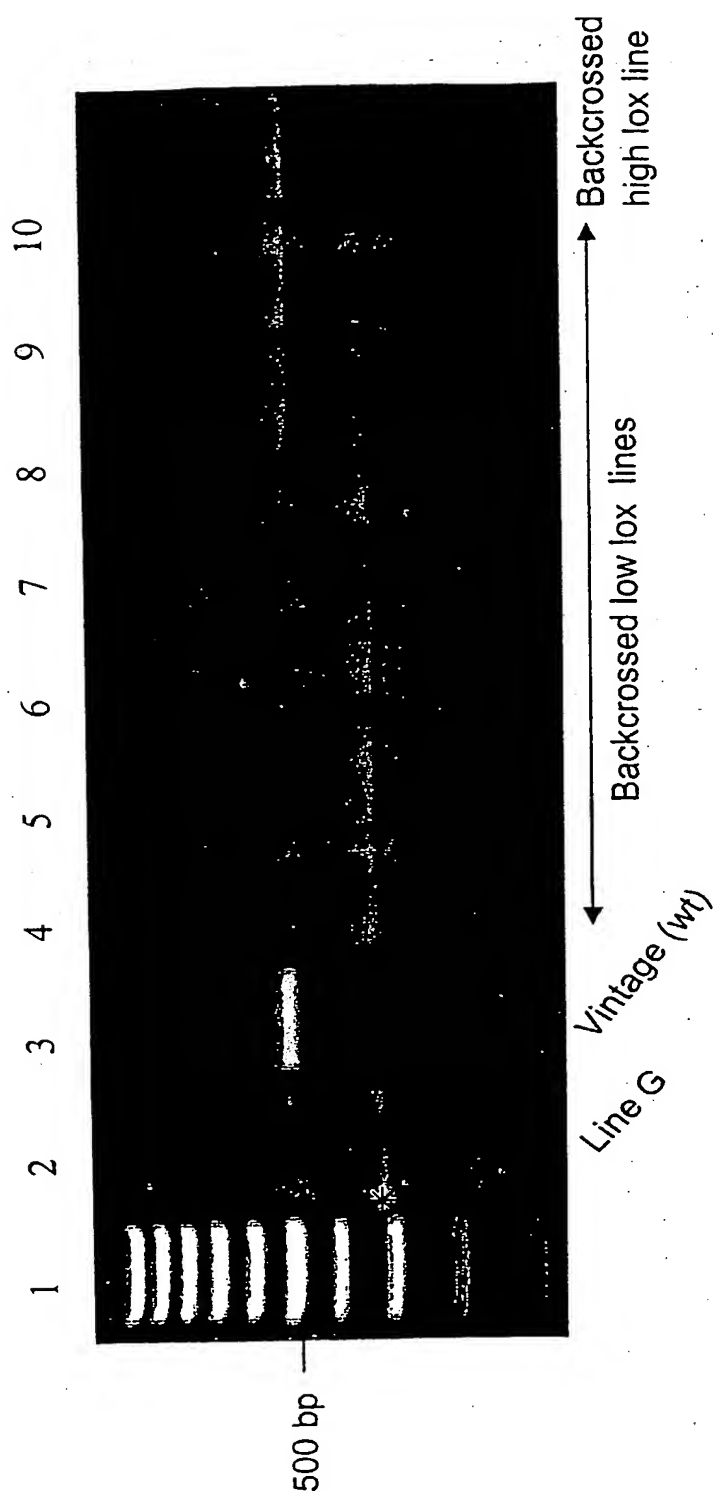


FIGURE 19

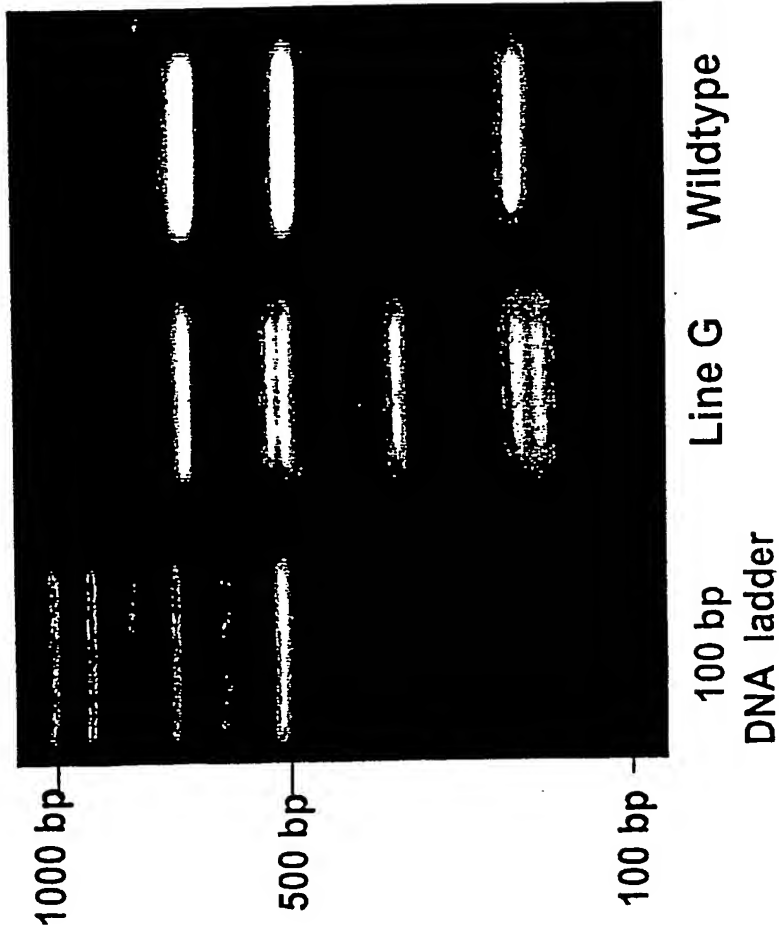


FIGURE 20

Backcrossing program for Line G to cv Alexis

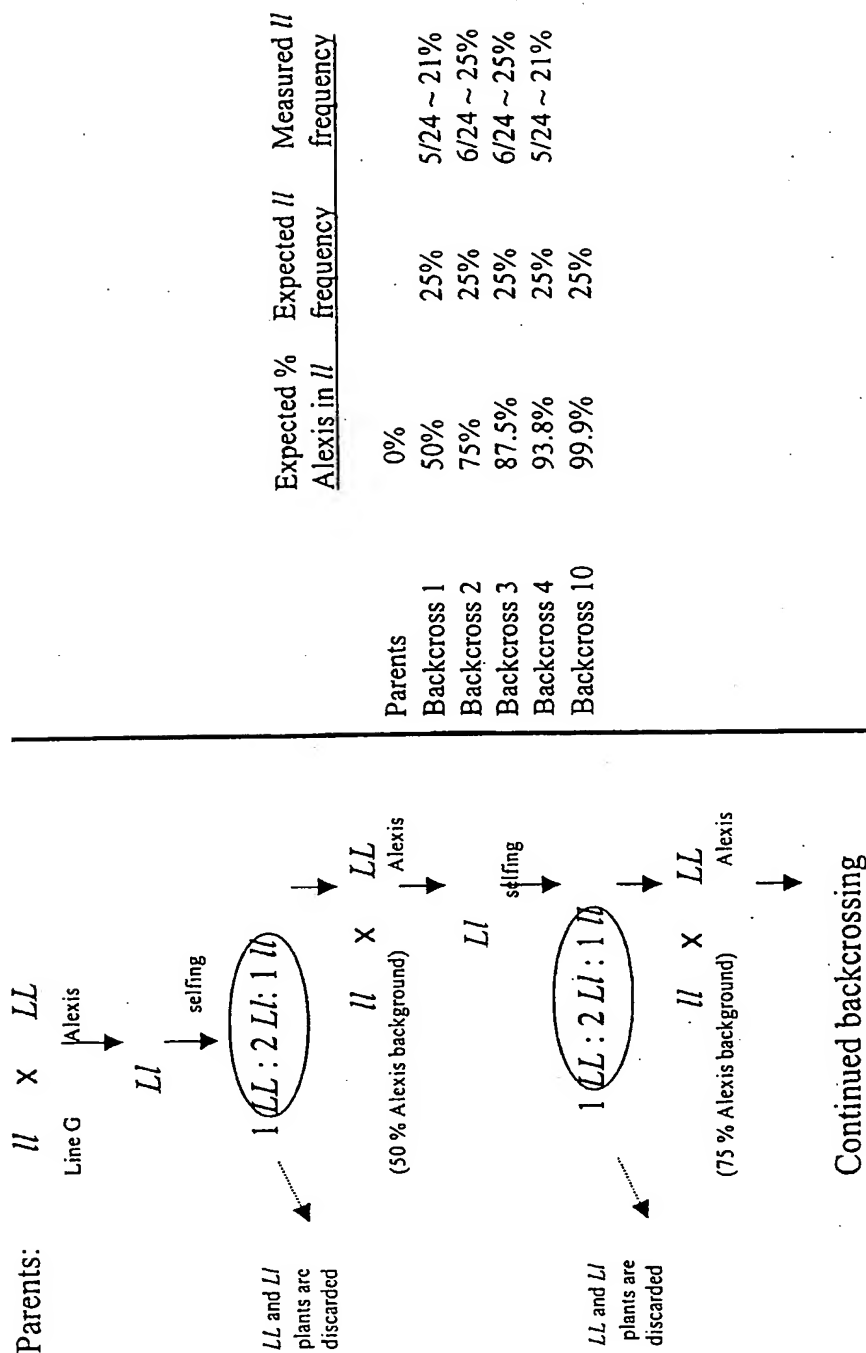


FIGURE 22 A
LIPOXYGENASE AMINO ACID SEQUENCE ALIGNMENT

```

1                               E1      H                               60
Gm1 MFS.....A.GHKIKGTVVLMRKNELE.....VNPDGSAV
Gm2 MFSVPGVSGILNRGG.GHKIKGTVVLMRKNVLDNFNSVADLTGKGVGGLIGTGLNVVGSTL
Gm3 M.....LGGLLHRG...HKIKGTVVLMRKNVLDVNSVT....SVGGIIGQGLDLVGSTL
Hv2 MLGVGGIVSDLTGGIRGAHLKGSVVLMRKNALDFN.....DFGAHVM
Hv1 ML.LGGLIDTLTGANKSARLKGTVVLMRKNVLDLN.....DFGATII
61      +      E2      ++ +      E3      +      +      E4 120
Gm1 DNLNAFLGRSVSLQLISATKADAH..GKGKVGKDTFLEG..INTSLPTLGAGESAFNIHF
Gm2 DNLTAFLGRSVALQLISATKPLAN..GKGKVGKDTFLEG..IIVSLPTLGAGESAFNIQF
Gm3 DTLTAFLGRSVSLQLISATKADAN..GKGKLGKATFLEG..IITSLPTLGAGQSAFKINF
Hv2 DGVTELLGRGVTCQLISSTNVHDHNGGRGKVGAEANLEQWLLPTNLPFITTTGENKFAVTF
Hv1 DGIGEFLGKGVTCQLISSTAVDQDNGGRGKVGAEAELEQWV..TSLPSLTGSGKFGLTF
121      + +      E5      E6      +      E7      E8 180
Gm1 EWD.GSMGIPGAFYIKNYMQVEFFLKSLTLEAISNQ.GTIRFVCNSWVYNTKLYKSVRIF
Gm2 EWD.ESMGIPGAFYIKNYMQVEFFLKSLTLEDVNPQ.GTIRFVCNSWVYNTKLYKSVRIF
Gm3 EWD.DGSGIPGAFYIKNFMQTEFFLVSLTLEDIPNH.GSIHFVCNSWIYNAKLKFSRIF
Hv2 DWSVDKLGVPGAIVKNNHASEFFLKTITLDNVPGR.GTIVFVANSWVYPQAKYRYNRVF
Hv1 DWEVEKLGVPGAIVVNNYHSSEFFLKTITLHDVPGRSGNLTFVANSWIYPAANYRYSRVF
181      +      +      H1      +      +      E      +      240
Gm1 FANHTYVPSETPAPLVSYREEELKSLRGNGT.GERKEYDRIYDYDVYNDLGNPDKSEKLA
Gm2 FANHTYVPSETPAALVGYYREEELKSLRGDGGK.GERKEHDRIYDYDVYNDLGNPDHGENFA
Gm3 FANQTYLPSETPAPLVKYREEELHNLRGDGT.GERKEWERIYDYDVYNDLGDPPDKGENHA
Hv2 FANDTYLPHQMPAALKPYRDELRNLRGDDQQGPYLDHDRVYRYDVYNDLGD.....S
Hv1 FANDTYLPSQMPAALKPYRDELRNLRGDDQQGPYQEHRIYRYDVYNDLGE.....G
241 ++      +      +      +      •••      • 300
Gm1 RPYLGGSSSTFPYPRRGRTGRGPTVTDPNTEKQ....GEVIFYPRDENLGHLSKSDATLEIG
Gm2 RPILGGSSSTHPYPRRGRTGRYPTRKQNSEKP....GEV.YVPRDENFGHLKSSDFLAYG
Gm3 RPYLGGNDTFPYPRRGRTGRKPTRKDPNSES....SNDVYLPRDEAFGHLKSSDFLTYG
Hv2 RDVLGGSKDLFPYPRRCRTGRKPSDSKPDHESRLLLLVQNVYVLRDELFGHLKQSDLLGYT
Hv1 RPILGGNSDHPYPRRGRTGRTERKPNASDPSLESRLSLL.EQIYVPRDEKFGHLKTSDFLGYS
•      H2      +      H3      ++      +      H4      +      360
Gm1 TKSLSQIVQPAFESAFDLKSTPIEFHSFQDVHDLYEGGIKLP....DVISTIIPLPVIK
Gm2 IKSLSQYVLPAPFESVFDLNFPTNEFDSFQDVLDHEGGIKLPT....EVISTIMPLPVVK
Gm3 LKSVSQNVLPPLQSAFDLNFPTREFDSFDEVHGLYSGGIKLP....DIISKISPLPVLK
Hv2 LKGWLDGIILAIRTYVDL..SPGEFDSFADIILKLYEGGIKLPNIPALEEVRKRFPPLQLVK
Hv1 IKAITQGILPAVRTYVDL..TPGEFDSFQDIINLYEGGIKLPKVALEELRKQFPPLQLIK
E10 +E11 + +      H6      ♦      +      E12      ++      420
Gm1 ELYRTDGQHILKFPQPHVVQVSQSAAWMTDEEFAREMIAGVNPCVIRGLEEFPPKSNLDP
Gm2 ELFRTDGEQVLKFPFPPHVIQVSKSAWMTDEEFAREMVAAGVNPCVIRGLQEFPPKSNLDP
Gm3 EIFRTDGEQALKFPFPPKVIQVSKSAWMTDEEFAREMLAGVNPNIIRCLKDFPPRSKLDSP
Hv2 DLI PKGGDFLLKLPKPEIIKVQKAWMTDEEFAREMLAGVNPMMIKRLTEFPKSTLDPS
Hv1 DLLPVGGDSLKLPVPHIIQENKQAWMTDEEFAREVLAGVNPVMTIRLTFEPKSSLDPS
+      +      +      H7      E13      H8      480
Gm1 IYGDQSSKITADSLD..LDGYTMDEALGSRRFLMDYHDI FMPYVRQINQLNSAKTYATR
Gm2 IYGEQTSKITADALD..LDGYTVDEALASRRFLMDYHDV FMPYIRRIQ.TYAKAYATR
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FIGURE 22 B

```

E14      +      E15      +      E16      +      +      H9      540
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Gm3 TILFLKNDGTLRPLATIELSLPHSAGDLSGAVSQVFLPADEG.VESSIWLAKAYVVVNDG
Hv2 TLLFLRGDGTLPVATIELSSPLIQGELTTAKSAVYTPQHAG.VEGWIWOLAKAYASVNDY
Hv1 TLFFLRGDGRLTPLATIELSEPIIQGGLTTAKSKVYTPVPSGSVEGWVWELAKAYVAVNDS

541 •      •      •      H9      +H10      +      •      H11      600+
Gm1 CYHQLMSHWLNTHAAMEPFVIAATHRHLSVLHPIYKLLTPHYRNNMNNALARQSLINAG
Gm2 CYHQLMSHWLNTHAVIEPFIIATNRHLSALHPIYKLLTPHYRDTMNNALARQSLINADG
Gm3 CYHQLVSHWLNTHAVVEPFIIATNRHLSVHPIYKLLHPHYRDTMNNGLARLSLVNDGG
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Hv1 GWHQLVSHWLNTHAVMEPFVISTNRHLSVTHPVHKLSPHYRDTMTINALARQTLINAGG

601 H12      H13      +H14      +      E      +      E      +660
Gm1 IIEETFLPSKYSVEMSSAVYKNWVFTDQALPADLIKRGVAIKDPSTPHGVRLIEDYPYA
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Hv2 VIEQTFWGRYSVEMSSAVYKHWNFTEQALPADLIKRGMAVEDASSPHKVRLLIKDYPYA
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661 H15      H16      H17      +      +      +      720
Gm1 ADGLEIWAIAIKTWVQYEVPLYARDDDVKNDSELQHWKKEAVEKGHGDLDKDPWPKLQT
Gm2 VDGLEIWAIAIKTWVQYEVSLYYARDDVKPDSELQQWKEAVEKGHGDLDKDPWPKLQT
Gm3 VDGLEIWAIAIKTWVHEYVFLYYKSDDTLREDPELQACWKEAVEKGHGDLDKDPWPKMQT
Hv2 TDGLAVWDAIEQWVSDYLTIIYYPNDGVLQGDVELQAWWKEVREVGHGDLDKDAAWPKMQT
Hv1 ADGLAIWAIAIEQYVSEYLAIIYYPNDGVLQGDTEVQAWWKEVREVGHGDLDKDAFWPKMQS

721      H18      *      •      •      +      •      +      +      H19      780
Gm1 LEDLVECLIIIIWIASALHAAVNFGQYFYGGILNNRPTASRRLLPEKGTPEYEEMINNHE
Gm2 IEELVEICTIIIIWTASALHAAVNFGQYFYGGILNNRPTSSRRLLPEKGTPEYEEMVKSHQ
Gm3 REELVEACIIIIWTASALHAAVNFGQYFYGGILNNRPTLSRRFMPEKGSAYEELRKNPQ
Hv2 VAELIKACATIIWTGSALHAAVNFGQYFYGGILNNRPTLSRRFMPEKGSAYEELRKNPQ
Hv1 VPELAKACTTIIWIGSALHAAVNFGQYFYAGFLNNRPTVSRRRMPEPGTEEYAELEDRPE

781 H20      H21 •      •      •      +      +      +      H22      840
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Gm3 KAYLKTITPKFQTLIDLSVIEIILSRHASDEVYLGQRDNPHWTSDSKALQAFQKFGNKLQ
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Hv1 RAFIHTITSQIQTIIIGVSLLEVLSKHSSDEVYLGQRDTPAWTSDAKALEAFKRFQKLEG

841      ++      +      +      +      +      +      +      895+
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Gm2 IEEKLARKNNDQSL.SNRLGPVQLPYTLLHPNSE.....GLTCRGIPNSISII
Gm3 IENKLSENNDEKLR.NRCGPVQMPYTLILLPSSE.....GLTFRGIPNSISII
Hv2 IEKQVVAMNSDPQLK.NRTGPAKFPYMLLYPNTSDHTGQAEGLTARGIPNSISII
Hv1 IESKVVGMNHDPELK.NRNGPAKFPYMLLYPNTSDHKGAAGLTAKGIPNSISII

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Gm1 SEQ ID NO:15
 Gm2 SEQ ID NO:16
 Gm3 SEQ ID NO:17
 Hv2 SEQ ID NO:18
 Hv1 SEQ ID NO:9

The numbering system shown above
 the sequence alignment does not
 correspond to the actual amino
 acid number of any lipoygenase
 sequence in the alignment.

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Doderer, Albert
Cameron-Mills, Verena
Skadhauge, Birgitte
Bech, Lene

<120> LOW LIPOXYGENASE 1 BARLEY

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33

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Gly Lys Gly val Thr Cys Gln Leu Ile Ser Ser Thr Ala val Asp Gln	50	55	60
Asp Asn Gly Gly Arg Gly Lys val Gly Ala Glu Ala Glu Leu Glu Gln	65	70	75
Trp val Thr Ser Leu Pro Ser Leu Thr Thr Gly Glu Ser Lys Phe Gly	85	90	95
Leu Thr Phe Asp Trp Glu val Glu Lys Leu Gly val Pro Gly Ala Ile	100	105	110
val val Asn Asn Tyr His Ser Ser Glu Phe Leu Leu Lys Thr Ile Thr	115	120	125
Leu His Asp val Pro Gly Arg Ser Gly Asn Leu Thr Phe val Ala Asn	130	135	140
Ser Trp Ile Tyr Pro Ala Ala Asn Tyr Arg Tyr Ser Arg val Phe Phe	145	150	155
Ala Asn Asp Thr Tyr Leu Pro Ser Gln Met Pro Ala Ala Leu Lys Pro	165	170	175
Tyr Arg Asp Asp Glu Leu Arg Asn Leu Arg Gly Asp Asp Gln Gln Gly	180	185	190
Pro Tyr Gln Glu His Asp Arg Ile Tyr Arg Tyr Asp val Tyr Asn Asp	195	200	205
Leu Gly Glu Gly Arg Pro Ile Leu Gly Gly Asn Ser Asp His Pro Tyr	210	215	220
Pro Arg Arg Gly Arg Thr Glu Arg Lys Pro Asn Ala Ser Asp Pro Ser	225	230	235
Leu Glu Ser Arg Leu Ser Leu Leu Glu Gln Ile Tyr val Pro Arg Asp	245	250	255
Glu Lys Phe Gly His Leu Lys Thr Ser Asp Phe Leu Gly Tyr Ser Ile	260	265	270
Lys Ala Ile Thr Gln Gly Ile Leu Pro Ala val Arg Thr Tyr val Asp	275	280	285
Thr Thr Pro Gly Glu Phe Asp Ser Phe Gln Asp Ile Ile Asn Leu Tyr	290	295	300
Glu Gly Gly Ile Lys Leu Pro Lys val Ala Ala Leu Glu Glu Leu Arg	305	310	315
Lys Gln Phe Pro Leu Gln Leu Ile Lys Asp Leu Leu Pro val Gly Gly	325	330	335
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SEQLIST.ST25

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Glu Ser Asn Arg Leu Tyr Ile Leu Asp His His Asp Arg Phe Met Pro		
420	425	430
Phe Leu Ile Asp Val Asn Asn Leu Pro Gly Asn Phe Ile Tyr Ala Thr		
435	440	445
Arg Thr Leu Phe Phe Leu Arg Gly Asp Gly Arg Leu Thr Pro Leu Ala		
450	455	460
Ile Glu Leu Ser Glu Pro Ile Ile Gln Gly Gly Leu Thr Thr Ala Lys		
465	470	475
Ser Lys Val Tyr Thr Pro Val Pro Ser Gly Ser Val Glu Gly Trp Val		
485	490	495
Trp Glu Leu Ala Lys Ala Tyr Val Ala Val Asn Asp Ser Gly Trp His		
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Gln Leu Val Ser His Trp Leu Asn Thr His Ala Val Met Glu Pro Phe		
515	520	525
Val Ile Ser Thr Asn Arg His Leu Ser Val Thr His Pro Val His Lys		
530	535	540
Leu Leu Ser Pro His Tyr Arg Asp Thr Met Thr Ile Asn Ala Leu Ala		
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Arg Gln Thr Leu Ile Asn Ala Gly Gly Ile Phe Glu Met Thr Val Phe		
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Pro Gly Lys Phe Ala Leu Gly Met Ser Ala Val Val Tyr Lys Asp Trp		
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Lys Phe Thr Glu Gln Gly Leu Pro Asp Asp Leu Ile Lys Arg Gly Met		
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Ala Val Glu Asp Pro Ser Ser Pro Tyr Lys Val Arg Leu Leu Val Ser		
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Asp Tyr Pro Tyr Ala Ala Asp Gly Leu Ala Ile Trp His Ala Ile Glu		
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Gln Tyr Val Ser Glu Tyr Leu Ala Ile Tyr Tyr Pro Asn Asp Gly Val		
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SEQLIST.ST25

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690 695 700

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Phe Leu Pro Asn Arg Pro Thr Val Ser Arg Arg Arg Met Pro Glu Pro
725 730 735

Gly Thr Glu Glu Tyr Ala Glu Leu Glu Arg Asp Pro Glu Arg Ala Phe
740 745 750

Ile His Thr Ile Thr Ser Gln Ile Gln Thr Ile Ile Gly Val Ser Leu
755 760 765

Leu Glu Val Leu Ser Lys His Ser Ser Asp Glu Leu Tyr Leu Gly Gln
770 775 780

Arg Asp Thr Pro Glu Trp Thr Ser Asp Pro Lys Ala Leu Glu Val Phe
785 790 795 800

Lys Arg Phe Ser Asp Arg Leu Val Glu Ile Glu Ser Lys Val Val Gly
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Met Asn His Asp Pro Glu Leu Lys Asn Arg Asn Gly Pro Ala Lys Phe
820 825 830

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acctcacctt cgtcgccaac tcatggatct accccgccgc caactaccga tacagccgcg 540

SEQLIST.ST25

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agttcggcca	cctcaagacg	tccgacttcc	tgggctactc	catcaaggcc	atcacgcagg	900
gcctcctgcc	ggccgtgcgc	acctacgtgg	acaccacccc	cggcgagtcc	gactccttcc	960
aggacatcat	caacctctat	gagggcggcga	tcaagctgcc	caagggtggcc	gccctggagg	1020
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ccctgcttaa	gctccccgtg	ccccacatca	tccaggagaa	caagcaggcg	tggaggaccg	1140
acgaggagtt	cgcacgggag	gtgctcgccg	gcgtcaaccc	ggcatgatc	acgcgtctca	1200
cggagttccc	gccaaaaagt	agtctggacc	ctagcaagtt	tggtgaccac	accagcacca	1260
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SEQLIST.ST25

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acagcatctc catctaattct aagccatcgg caaccatgga tgaataaagg gcgttcgcca 2700
cgtacgaaac ttgtcgagag attggtgtag tgtgtgtctg tgacagtact atgtcagcag 2760
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<210> 11

<211> 4663

<212> DNA

<213> Hordeum vulgare

<220>

<221> variation

<222> (2346)..(2348)

<223> "n" is a, c, t, or g encoding an acidic, basic, or polar amino ac
i

```

<400> 11
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gcgccacacc accgggaccg gaccgggacc ggcctataaa ttgcccggac cgagctgcaa 240
gcagctcctc acacacactc acgcaacaca catccatctt cactgaaaag tgaaaaacag 300
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cgggtggtgct catgcgcaag aacgtgctgg acctcaacga cttcggcgcc accatcatcg 480
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tcgaccaagg taatcactac cctcctccgg ccttctcctc tgtttacaag atatagtatt 600
tctttcgtgt gggccggcgg ccatggatgg atggatgtgt ctggatcggc taaagaagat 660
aggatagcta gccctggccg gtcgtcttta cctgagcatg ggcataatgcc atcgaaaaaa 720
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..SEQLIST.ST25

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cagcaggcta taaacagttg tcatcaaaaa caacgctgga agagagagag aaggaaggaa	960
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tggtggtgca gacaacggcg gtcgcgggaa ggtgggcgcg gaggcggagc tggagcagtg	1140
ggtgacgagc ctgccgtcgc tgacgacggg ggagtccaag ttcggcctca ccttcgactg	1200
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caacgacgtg cgtggatttt cctctacttt cctctccttt cattttcacc gccttcgtca	1440
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cttagcagta cgtacattat ttattccgtg ttggtagaaa aggatatggt ttggtgcaga	1560
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aactgttgca cggatccacc cacatgattg ttttcatgaa taaacttttt aaggatgtgt	1680
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SEQLIST.ST25

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SEQLIST.ST25

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gcagttgctc ttttagccga ataaataaag cagatttgct tcc 4663

<210> 12

<211> 862

<212> PRT

<213> Hordeum vulgare

<220>

<221> variation

<222> (368)..(368)

<223> "xaa" is an acidic, basic, or polar amino acid

<400> 12

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Leu Asn Asp Phe Gly Ala Thr Ile Ile Asp Gly Ile Gly Glu Phe Leu
35 40 45
Gly Lys Gly Val Thr Cys Gln Leu Ile Ser Ser Thr Ala Val Asp Gln
50 55 60
Asp Asn Gly Gly Arg Gly Lys Val Gly Ala Glu Ala Glu Leu Glu Gln
65 70 75 80
Trp Val Thr Ser Leu Pro Ser Leu Thr Thr Gly Glu Ser Lys Phe Gly
85 90 95
Leu Thr Phe Asp Trp Glu Val Glu Lys Leu Gly Val Pro Gly Ala Ile
100 105 110
Val Val Asn Asn Tyr His Ser Ser Glu Phe Leu Leu Lys Thr Ile Thr
115 120 125
Leu His Asp Val Pro Gly Arg Ser Gly Asn Leu Thr Phe Val Ala Asn
130 135 140
Ser Trp Ile Tyr Pro Ala Ala Asn Tyr Arg Tyr Ser Arg Val Phe Phe
145 150 155 160
Ala Asn Asp Thr Tyr Leu Pro Ser Gln Met Pro Ala Ala Leu Lys Pro
165 170 175
Tyr Arg Asp Asp Glu Leu Arg Asn Leu Arg Gly Asp Asp Gln Gln Gly
180 185 190

SEQLIST.ST25

Pro Tyr Gln Glu His Asp Arg Ile Tyr Arg Tyr Asp Val Tyr Asn Asp
 195 200 205
 Leu Gly Glu Gly Arg Pro Ile Leu Gly Gly Asn Ser Asp His Pro Tyr
 210 215 220
 Pro Arg Arg Gly Arg Thr Glu Arg Lys Pro Asn Ala Ser Asp Pro Ser
 225 230 235 240
 Leu Glu Ser Arg Leu Ser Leu Leu Glu Gln Ile Tyr Val Pro Arg Asp
 245 250 255
 Glu Lys Phe Gly His Leu Lys Thr Ser Asp Phe Leu Gly Tyr Ser Ile
 260 265 270
 Lys Ala Ile Thr Gln Gly Ile Leu Pro Ala Val Arg Thr Tyr Val Asp
 275 280 285
 Thr Thr Pro Gly Glu Phe Asp Ser Phe Gln Asp Ile Ile Asn Leu Tyr
 290 295 300
 Glu Gly Gly Ile Lys Leu Pro Lys Val Ala Ala Leu Glu Glu Leu Arg
 305 310 315 320
 Lys Gln Phe Pro Leu Gln Leu Ile Lys Asp Leu Leu Pro Val Gly Gly
 325 330 335
 Asp Ser Leu Leu Lys Leu Pro Val Pro His Ile Ile Gln Glu Asn Lys
 340 345 350
 Gln Ala Trp Arg Thr Asp Glu Glu Phe Ala Arg Glu Val Leu Ala Xaa
 355 360 365
 Val Asn Pro Val Met Ile Thr Arg Leu Thr Glu Phe Pro Pro Lys Ser
 370 375 380
 Ser Leu Asp Pro Ser Lys Phe Gly Asp His Thr Ser Thr Ile Thr Ala
 385 390 395 400
 Glu His Ile Glu Lys Asn Leu Glu Gly Leu Thr Val Gln Gln Ala Leu
 405 410 415
 Glu Ser Asn Arg Leu Tyr Ile Leu Asp His His Asp Arg Phe Met Pro
 420 425 430
 Phe Leu Ile Asp Val Asn Asn Leu Pro Gly Asn Phe Ile Tyr Ala Thr
 435 440 445
 Arg Thr Leu Phe Phe Leu Arg Gly Asp Gly Arg Leu Thr Pro Leu Ala
 450 455 460
 Ile Glu Leu Ser Glu Pro Ile Ile Gln Gly Gly Leu Thr Thr Ala Lys
 465 470 475 480
 Ser Lys Val Tyr Thr Pro Val Pro Ser Gly Ser Val Glu Gly Trp Val
 485 490 495
 Trp Glu Leu Ala Lys Ala Tyr Val Ala Val Asn Asp Ser Gly Trp His
 500 505 510
 Gln Leu Val Ser His Trp Leu Asn Thr His Ala Val Met Glu Pro Phe
 515 520 525

SEQLIST.ST25

Val	Ile	Ser	Thr	Asn	Arg	His	Leu	Ser	Val	Thr	His	Pro	Val	His	Lys
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Leu	Leu	Ser	Pro	His	Tyr	Arg	Asp	Thr	Met	Thr	Ile	Asn	Ala	Leu	Ala
545					550					555					560
Arg	Gln	Thr	Leu	Ile	Asn	Ala	Gly	Gly	Ile	Phe	Glu	Met	Thr	Val	Phe
				565					570					575	
Pro	Gly	Lys	Phe	Ala	Leu	Gly	Met	Ser	Ala	Val	Val	Tyr	Lys	Asp	Trp
			580					585					590		
Lys	Phe	Thr	Glu	Gln	Gly	Leu	Pro	Asp	Asp	Leu	Ile	Lys	Arg	Gly	Met
		595					600					605			
Ala	Val	Glu	Asp	Pro	Ser	Ser	Pro	Tyr	Lys	Val	Arg	Leu	Leu	Val	Ser
	610					615					620				
Asp	Tyr	Pro	Tyr	Ala	Ala	Asp	Gly	Leu	Ala	Ile	Trp	His	Ala	Ile	Glu
625					630					635					640
Gln	Tyr	Val	Ser	Glu	Tyr	Leu	Ala	Ile	Tyr	Tyr	Pro	Asn	Asp	Gly	Val
				645					650					655	
Leu	Gln	Gly	Asp	Thr	Glu	Val	Gln	Ala	Trp	Trp	Lys	Glu	Thr	Arg	Glu
			660					665					670		
Val	Gly	His	Gly	Asp	Leu	Lys	Asp	Ala	Pro	Trp	Trp	Pro	Lys	Met	Gln
		675					680					685			
Ser	Val	Pro	Glu	Leu	Ala	Lys	Ala	Cys	Thr	Thr	Ile	Ile	Trp	Ile	Gly
	690					695					700				
Ser	Ala	Leu	His	Ala	Ala	Val	Asn	Phe	Gly	Gln	Tyr	Pro	Tyr	Ala	Gly
705					710					715					720
Phe	Leu	Pro	Asn	Arg	Pro	Thr	Val	Ser	Arg	Arg	Arg	Met	Pro	Glu	Pro
				725					730					735	
Gly	Thr	Glu	Glu	Tyr	Ala	Glu	Leu	Glu	Arg	Asp	Pro	Glu	Arg	Ala	Phe
			740					745					750		
Ile	His	Thr	Ile	Thr	Ser	Gln	Ile	Gln	Thr	Ile	Ile	Gly	Val	Ser	Leu
		755					760					765			
Leu	Glu	Val	Leu	Ser	Lys	His	Ser	Ser	Asp	Glu	Leu	Tyr	Leu	Gly	Gln
	770					775					780				
Arg	Asp	Thr	Pro	Glu	Trp	Thr	Ser	Asp	Pro	Lys	Ala	Leu	Glu	Val	Phe
785					790					795					800
Lys	Arg	Phe	Ser	Asp	Arg	Leu	Val	Glu	Ile	Glu	Ser	Lys	Val	Val	Gly
				805					810					815	
Met	Asn	His	Asp	Pro	Glu	Leu	Lys	Asn	Arg	Asn	Gly	Pro	Ala	Lys	Phe
			820					825					830		
Pro	Tyr	Met	Leu	Leu	Tyr	Pro	Asn	Thr	Ser	Asp	His	Lys	Gly	Ala	Ala
		835					840					845			
Ala	Gly	Leu	Thr	Ala	Lys	Gly	Ile	Pro	Asn	Ser	Ile	Ser	Ile		
	850					855					860				

SEQLIST.ST25

<210> 13

<211> 20

<212> DNA

<213> Hordeum vulgare

<400> 13

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20

<210> 14

<211> 20

<212> DNA

<213> Hordeum vulgare

<400> 14

cagactactt tttggcggga

20

<210> 15

<211> 839

<212> PRT

<213> Glycine max

<400> 15

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 Asn Ala Phe Leu Gly Arg Ser Val Ser Leu Gln Leu Ile Ser Ala Thr
 35 40 45
 Lys Ala Asp Ala His Gly Lys Gly Lys Val Gly Lys Asp Thr Phe Leu
 50 55 60
 Glu Gly Ile Asn Thr Ser Leu Pro Thr Leu Gly Ala Gly Glu Ser Ala
 65 70 75 80
 Phe Asn Ile His Phe Glu Trp Asp Gly Ser Met Gly Ile Pro Gly Ala
 85 90 95
 Phe Tyr Ile Lys Asn Tyr Met Gln Val Glu Phe Phe Leu Lys Ser Leu
 100 105 110
 Thr Leu Glu Ala Ile Ser Asn Gln Gly Thr Ile Arg Phe Val Cys Asn
 115 120 125

SEQLIST.ST25

Ser Trp Val Tyr Asn Thr Lys Leu Tyr Lys Ser Val Arg Ile Phe Phe
 130 135 140
 Ala Asn His Thr Tyr Val Pro Ser Glu Thr Pro Ala Pro Leu Val Ser
 145 150 155 160
 Tyr Arg Glu Glu Glu Leu Lys Ser Leu Arg Gly Asn Gly Thr Gly Glu
 165 170 175
 Arg Lys Glu Tyr Asp Arg Ile Tyr Asp Tyr Asp Val Tyr Asn Asp Leu
 180 185 190
 Gly Asn Pro Asp Lys Ser Glu Lys Leu Ala Arg Pro Val Leu Gly Gly
 195 200 205
 Ser Ser Thr Phe Pro Tyr Pro Arg Arg Gly Arg Thr Gly Arg Gly Pro
 210 215 220
 Thr Val Thr Asp Pro Asn Thr Glu Lys Gln Gly Glu Val Phe Tyr Val
 225 230 235 240
 Pro Arg Asp Glu Asn Leu Gly His Leu Lys Ser Lys Asp Ala Leu Glu
 245 250 255
 Ile Gly Thr Lys Ser Leu Ser Gln Ile Val Gln Pro Ala Phe Glu Ser
 260 265 270
 Ala Phe Asp Leu Lys Ser Thr Pro Ile Glu Phe His Ser Phe Gln Asp
 275 280 285
 Val His Asp Leu Tyr Glu Gly Gly Ile Lys Leu Pro Arg Asp Val Ile
 290 295 300
 Ser Thr Ile Ile Pro Leu Pro Val Ile Lys Glu Leu Tyr Arg Thr Asp
 305 310 315 320
 Gly Gln His Ile Leu Lys Phe Pro Gln Pro His Val Val Gln Val Ser
 325 330 335
 Gln Ser Ala Trp Met Thr Asp Glu Glu Phe Ala Arg Glu Met Ile Ala
 340 345 350
 Gly Val Asn Pro Cys Val Ile Arg Gly Leu Glu Glu Phe Pro Pro Lys
 355 360 365
 Ser Asn Leu Asp Pro Ala Ile Tyr Gly Asp Gln Ser Ser Lys Ile Thr
 370 375 380
 Ala Asp Ser Leu Asp Leu Asp Gly Tyr Thr Met Asp Glu Ala Leu Gly
 385 390 395 400
 Ser Arg Arg Leu Phe Met Leu Asp Tyr His Asp Ile Phe Met Pro Tyr
 405 410 415
 Val Arg Gln Ile Asn Gln Leu Asn Ser Ala Lys Thr Tyr Ala Thr Arg
 420 425 430
 Thr Ile Leu Phe Leu Arg Glu Asp Gly Thr Leu Lys Pro Val Ala Ile
 435 440 445
 Glu Leu Ser Leu Pro His Ser Ala Gly Asp Leu Ser Ala Ala Val Ser
 450 455 460

SEQLIST.ST25

Gln Val Val Leu Pro Ala Lys Glu Gly Val Glu Ser Thr Ile Trp Leu
 465 470 475 480
 Leu Ala Lys Ala Tyr val Ile val Asn Asp Ser Cys Tyr His Gln Leu
 485 490 495
 Met Ser His Trp Leu Asn Thr His Ala Ala Met Glu Pro Phe val Ile
 500 505 510
 Ala Thr His Arg His Leu Ser Val Leu His Pro Ile Tyr Lys Leu Leu
 515 520 525
 Thr Pro His Tyr Arg Asn Asn Met Asn Ile Asn Ala Leu Ala Arg Gln
 530 535 540
 Ser Leu Ile Asn Ala Asn Gly Ile Ile Glu Thr Thr Phe Leu Pro Ser
 545 550 555 560
 Lys Tyr Ser val Glu Met Ser Ser Ala val Tyr Lys Asn Trp val Phe
 565 570 575
 Thr Asp Gln Ala Leu Pro Ala Asp Leu Ile Lys Arg Gly val Ala Ile
 580 585 590
 Lys Asp Pro Ser Thr Pro His Gly val Arg Leu Leu Ile Glu Asp Tyr
 595 600 605
 Pro Tyr Ala Ala Asp Gly Leu Glu Ile Trp Ala Ala Ile Lys Thr Trp
 610 615 620
 val Gln Glu Tyr val Pro Leu Tyr Tyr Ala Arg Asp Asp Asp val Lys
 625 630 635 640
 Asn Asp Ser Glu Leu Gln His Trp Trp Lys Glu Ala val Glu Lys Gly
 645 650 655
 His Gly Asp Leu Lys Asp Lys Pro Trp Trp Pro Lys Leu Gln Thr Leu
 660 665 670
 Glu Asp Leu val Glu val Cys Leu Ile Ile Ile Trp Ile Ala Ser Ala
 675 680 685
 Leu His Ala Ala val Asn Phe Gly Gln Tyr Pro Tyr Gly Gly Leu Ile
 690 695 700
 Met Asn Arg Pro Thr Ala Ser Arg Arg Leu Leu Pro Glu Lys Gly Thr
 705 710 715 720
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SEQLIST.ST25

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 850 855 860

INTERNATIONAL SEARCH REPORT

International Application No.

PCT/IB 00/02045

A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 C12N9/02 C12N15/82 A01H5/10

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C12N A01H

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the International search (name of data base and, where practical, search terms used)

BIOSIS, EPO-Internal, FSTA, WPI Data, PAJ, CAB Data, BIOTECHNOLOGY ABS, SCISEARCH
CHEM ABS Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	CHEMICAL ABSTRACTS, vol. 119, no. 23, 6 December 1993 (1993-12-06) Columbus, Ohio, US; abstract no. 248220m, KOWAKA, M.: "Malting barley improvement for brewing" page 828; column 2; XP002175449 abstract & NIPPON JOZO KYOKAISHI, vol. 88, no. 8, 1993, pages 574-581, --- -/--	1

☒ Further documents are listed in the continuation of box C.☒ Patent family members are listed in annex.

* Special categories of cited documents:

- *A* document defining the general state of the art which is not considered to be of particular relevance
- *E* earlier document but published on or after the international filing date
- *L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- *O* document referring to an oral disclosure, use, exhibition or other means
- *P* document published prior to the international filing date but later than the priority date claimed

- *T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- *X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- *Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- *&* document member of the same patent family

Date of the actual completion of the international search

23 August 2001

Date of mailing of the international search report

06/09/2001

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2
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Fax: (+31-70) 340-3016

Authorized officer

De Kok, A

INTERNATIONAL SEARCH REPORT

Inter id Application No

PCT/IB 00/02045

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>WAN HENG WANG ET AL: "Molecular basis of a null mutation in soybean lipoxygenase 2: substitution of glutamine for an iron-ligand histidine." PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES, vol. 91, no. 13, 1994, pages 5828-5832, XP002175448 WASHINGTON US abstract</p>	1
X	<p>MCELROY D ET AL: "WHAT'S BREWING IN BARLEY BIOTECHNOLOGY?" BIO/TECHNOLOGY, NEW YORK, US, vol. 13, no. 3, 1995, pages 245-249, XP002024712 ISSN: 0733-222X cited in the application page 247, column 2, last paragraph</p>	11
Y	<p>DROST B W ET AL: "FLAVOR STABILITY" JOURNAL OF THE AMERICAN SOCIETY OF BREWING CHEMISTS, vol. 48, no. 4, 1990, pages 124-131, XP000926610 ISSN: 0361-0470 cited in the application</p>	11-15
A	<p>the whole document, especially pages 129-130</p>	1,21-27
Y	<p>WO 97 13851 A (PURDUE RESEARCH FOUNDATION (US)) 17 April 1997 (1997-04-17) abstract</p>	11-15
A	<p>VAN MECHELEN JAN R ET AL: "Molecular characterization of two lipoxygenases from barley." PLANT MOLECULAR BIOLOGY, vol. 39, no. 6, April 1999 (1999-04), pages 1283-1298, XP001015731 ISSN: 0167-4412 abstract; figure 1</p>	1-10

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.2

Present claim 1 relate to a barley plant defined by reference to desirable characteristic or property, namely characterized by a reduction or absence of lipoxygenase activity.

The claims cover all plants having this characteristic or property, whereas the application provides support within the meaning of Article 6 PCT and/or disclosure within the meaning of Article 5 PCT for only a very limited number of such plants. In the present case, the claims so lack support, and the application so lacks disclosure, that a meaningful search over the whole of the claimed scope is impossible.

Independent of the above reasoning, the claims also lack clarity (Article 6 PCT). An attempt is made to define the plant by reference to a result to be achieved. Again, this lack of clarity in the present case is such as to render a meaningful search over the whole of the claimed scope impossible. Consequently, the search has been carried out for those parts of the claims which appear to be clear, supported and disclosed, namely those parts relating to the subject-matter of claim 8 and claims dependent thereof.

Present claims 6 and 7 relate to an extremely large number of possible barley plants carrying a mutated *lox-1* gene. Support within the meaning of Article 6 PCT and/or disclosure within the meaning of Article 5 PCT is to be found, however, for only ONE mutated *lox-1* gene.

In the present case, the claims so lack support, and the application so lacks disclosure, that a meaningful search over the whole of the claimed scope is impossible. Consequently, the search has been carried out for those parts of the claims which appear to be supported and disclosed, namely those parts relating to the subject-matter of claim 8 and claims dependent thereof.

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

INTERNATIONAL SEARCH REPORT

information on patent family members

Intern Application No

PCT/IB 00/02045

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
W0 9713851 A	17-04-1997	AU 7443596 A	30-04-1997
		CA 2234107 A	17-04-1997
		EP 0859836 A	26-08-1998
<hr/>			

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